Biophysical Chemistry 212 (2016) 17–21

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Review

Protein folding: Vexing debates on a fundamental problem

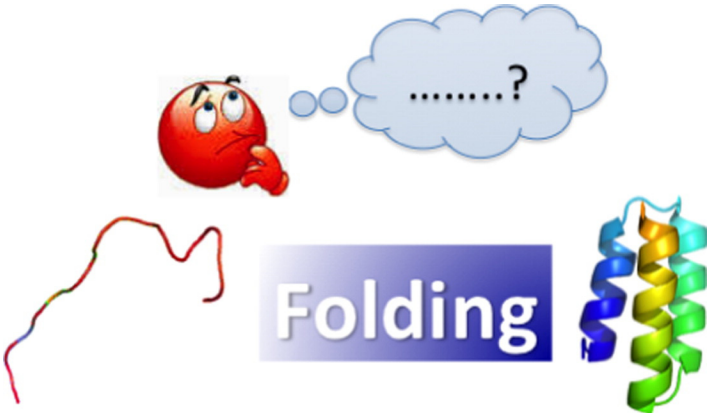
Stefano Gianni [a,b,*], Per Jemth [c,*]

[a] Istituto Pasteur, Fondazione Cenci Bolognetti and Istituto di Biologia e Patologia Molecolari del CNR, Dipartimento di Scienze Biochimiche "A. Rossi Fanelli" Sapienza, University of Rome, 00185 Rome, Italy
[b] Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom
[c] Department of Medical Biochemistry and Microbiology, Uppsala University, BMC Box 582, SE-75123 Uppsala, Sweden

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



ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 25 February 2016
Accepted 7 March 2016
Available online 17 March 2016

Keywords:
Protein folding
Kinetics
Mutagenesis
Denatured state
Transition state
Transition path time

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.

Contents

* Corresponding authors.
E-mail addresses: Stefano.Gianni@uniroma1.it (S. Gianni), Per.Jemth@imbim.uu.se (P. Jemth).

http://dx.doi.org/10.1016/j.bpc.2016.03.001
0301-4622/

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 refereed 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

work has been devoted to characterizing its structure. The most powerful experimental technique to solve the structure of the transition state for folding is the so-called Φ value analysis. [41,42] This methodology is based on the synergy between mutagenesis and kinetics and relies on measuring the effect of amino acid side chain perturbations on the free energies of the folded and transition states, respectively. By normalizing the change in activation free energy upon mutation to that of the folded state it is possible to obtain a structural index, named Φ. Quantitatively, the Φ value for folding is defined as:

$$\Phi = \frac{\Delta\Delta G_{D-TS}}{\Delta\Delta G_{D-N}} \quad (1)$$

$\Phi = 1$ indicates that the site of mutation is fully structured in the transition state, $\Phi = 0$ indicates that the site of mutation is as unstructured as the denatured state *D*.

Ever since its introduction, the Φ value analysis has attracted a great interest, but also been challenged from different angles. In particular, two types of debates arose: i) the perturbation introduced by the mutation distorts or de-routes the genuine folding pathway of the wild type system [43,44]; ii) the analysis of the observed kinetic parameters is not accurate enough to provide reliable values [45,46]. Most of the criticisms were rebutted by Fersht and co-workers [47], who have also provided guidelines on how to choose the mutations and how to perform the error analysis of Φ values [48]. Here, we focus on one particular criticism.

In a recent analysis, Naganathan and Muñoz reassessed a large database of different mutants and questioned the importance of Φ values [49]. In response to their points, we have previously used the comparison of the folding between different homologous protein systems to demonstrate the robustness of Φ values (Φ-Φ plots) [50]; a finding that reinforces their significance in protein folding studies. In this review, we will consider the point of Naganathan and Muñoz from a different perspective. They analyzed more than 800 mutations in 24 different proteins as a single data set and concluded that "all data were consistent with a single Φ value (0.24) with accuracy comparable to experimental precision, suggesting that the structural information in conventional Φ values is low". That is – because the Φ value represents a two-point slope of the change in the activation free energy versus the change in ground state free energy upon mutation, if all the mutants (when considered together) are *consistent* with a single slope, it follows that little structural information is contained in the Φ values. In analogy to this analysis, we report in Fig. 1 the dependence of the time in seconds versus the meters of all the world records in running for 100 m up to marathon (42,195 m), for both women and men. It may be observed that all the data are consistent with a single slope of 5.4 ± 0.07 m/s. Because the speed of a runner represents the two-point slope of the meters of a given athletic discipline versus time, a paradoxical analogy of the analysis performed by Naganathan and Muñoz may suggest that *all the runners (from 100 m to marathon), were consistent with a single speed, suggesting that the information in conventional speed measurement is low.* Of particular interest, the personal best times for one of us fall relatively close to the line suggesting a speed which could challenge Usain Bolt. Obviously, the linearity is an artefact of the scaling and each point holds precise and accurate information. Similarly, whilst each Φ value is robust and reliable, when all the mutants are considered together, an apparent (artificial) linearity arises.

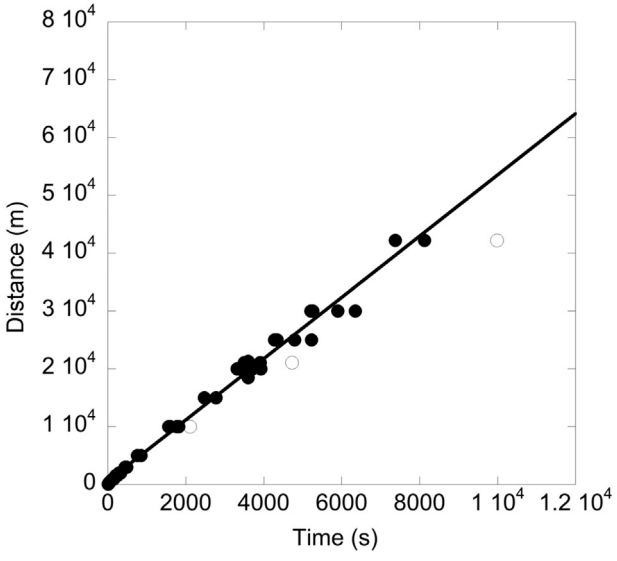


Fig. 1. Dependence of distance over time for running world records for both men and women. The line is the best fit to a linear equation. Open circles refer to the best personal records for 10,000 m, half marathon and marathon for one of the authors of the present paper.

We suggest that the average Φ value of 0.24 is in fact the consequence of the generality of the so-called nucleation-condensation mechanism in protein folding [51–53]. By following this model, whilst folding is driven by nucleation of specific residues, formation of the nucleus can only occur if a significant fraction of the overall structure is in a conformation approximating the native one. Thus, formation of the nucleus (nucleation) is coupled with a more general formation of structure (condensation), giving rise to a diffuse transition state, resembling a distorted version of the native state. Therefore, because the transition state is similar in structure to the native state, with a robust native-like structural content, a global analysis of the mutational variants will return an overall linear behavior. But the discussion will go on.

4. The transition path

Our current understanding of chemical reactions is based on Arrhenius theory on the transition state [54]. From this view, the whole concept of kinetics has a probabilistic nature - molecules jump *quasi* instantaneously between thermodynamic wells, with a probability over time that is proportional to the height of the intervening free-energy barrier, with the resulting apparent kinetics for a monomolecular reaction conforming to a single exponential decay. A breakdown of such an exponential decay may occur in the absence of a barrier [55] (the so called 'downhill folding'), a controversial scenario that has been extensively discussed previously [56–61]. The time that is required for a molecule to transit from one state to another is defined as the 'transition path time' and represents a quantity that, until very recently, has escaped experimental detection, for any molecular process in solution.

In 2009, by employing smFRET and analysing photon-by-photon trajectories of protein folding and unfolding, Eaton and co-workers measured for the first time the upper bound for the transition path time of a chemical reaction, the folding of GB1 [62]. A few years later, this ground-breaking work was continued by measuring the transition path time of GB1 in comparison to that of a WW domain and of α3d [63,64], by quantifying the influence of viscosity in barrier crossing [64] and by determining the role of specific non-native interactions in the dynamics of barrier crossing, rather than in altering the height of free-energy barriers [65]. The experimental work was compared with very long molecular dynamics simulations (in the μs to 1 ms time range) carried out by Shaw and co-workers on the most powerful computer in the world, Anton 2, built specifically to study protein folding [66].

The detection and characterization of transition path times is of outstanding interest and takes our understanding of protein folding to a new level. Over and above the molecular details of the reactions, which were exhaustively discussed in the original works, we wish to

briefly discuss here two observations that are particularly surprising – i) the transition path time of different proteins is rather conserved and fast folders display a transition path time comparable to slow folders; ii) the transition path time is relatively long, being in the order of 1 μs, that is, on the same time scale as the fastest folding protein domains. Attila Szabo provided a quantitative method to extract the transition path times from the analysis of single molecule trajectories, and reported an explanation of the observed robustness of the transition path time between different protein systems [62]. By describing the diffusive motion of chemical particles over a barrier, he demonstrated that the transition path time is proportional to the logarithm of the activation free energy, which minimizes the observed variations in transition path times between fast and slow-folding proteins.

One of the most interesting approaches to override the limitations of computer power in molecular dynamics simulations has been represented by distributed computing [67,68]. Instead of running a single long simulation to achieve at best a few tens of μs with the top-notch computers (a time scale still very far from the majority of proteins, folding in the ms to second time scale), thousands and thousands of very short simulations were performed on screen savers of personal computers around the world. In theory, even if the simulations are very short compared to the time scale of the folding reaction, by performing a sufficiently high number of simulations there would be a probability for a few of them to converge. Now back to the transition path time: The observation that the transition path time, even for very simple systems, may be as long as 1 μs, seems to represent an additional complication to distributed computing and for any very short simulation in the ns time range. In fact, whilst it is clear that the transition path time measured by Eaton and co-workers is an average value and more fast events are theoretically possible, we note that the probability of distributed computing to pick a really converging simulation is in fact probably much smaller than previously estimated and long simulations, such as those performed by Shaw and co-workers [65,66], are needed to describe a complex reaction such as protein folding.

5. Conclusions

The improvement of innovative experimental techniques, the development of super computers as well as the accumulation of a large amount of mutational data on different proteins have contributed to increase our knowledge of protein folding tremendously. Nevertheless, many questions are still open and new debates are arising. Furthermore, considerable efforts are still needed to investigate some issues that are relatively unexplored. Strikingly, the vast majority of the work on protein folding, including our own, still focuses on simple systems and our understanding of the folding of multi-domain large (some would argue “real”) proteins is very limited and only few studies may be found, see for example [69–71]. If one compares the complexity of the protein systems used by Richards and Anfinsen with most of the modern work on protein folding, it would appear that the field is going in the reverse direction, towards smaller proteins. Continued efforts are therefore needed to bridge the gap between our knowledge on simple protein domains and large complex proteins, preferably in a cellular environment [72,73] or in the context of the interaction of the nascent chain with the ribosome [74,75].

Acknowledgments

This work was supported by the Swedish Research Council NT [grant number 2012- 5096] (to P. J.), by the Italian Ministero dell'Istruzione dell'Universita` e della Ricerca (Progetto di Interesse ‘Invecchiamento’ to S.G.) and by Sapienza Università di Roma (C26A155S48 to S.G.)

References

[1] Editorial, So much more to know Science, 309 2005, pp. 78–102.
[2] V. Daggett, A.R. Fersht, Is there a unifying mechanism for protein folding? Trends Biochem. Sci. 28 (1) (2003) 18–25.
[3] A. Perez, J.A. Morrone, C. Simmerling, K.A. Dill, Advances in free-energy-based simulations of protein folding and ligand binding, Curr. Opin. Struct. Biol. 36 (2016) 25–31.
[4] P.G. Wolynes, Energy landscapes and solved protein-folding problems, Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 363 (2005) 453–464.
[5] A.A. Nickson, B.G. Wensley, J. Clarke, Take home lessons from studies of related proteins, Curr. Opin. Struct. Biol. 23 (2013) 66–74.
[6] A.R. Fersht, From the first protein structures to our current knowledge of protein folding: delights and scepticisms, Nat. Rev. Mol. Cell Biol. 9 (2008) 650–654.
[7] A.I. Bartlett, S.E. Radford, An expanding arsenal of experimental methods yields an explosion of insights into protein folding mechanisms, Nat. Struct. Mol. Biol. 16 (2009) 582–588.
[8] C. Tanford, Protein denaturation, Adv. Protein Chem. 24 (1968) 1–95.
[9] S.E. Jackson, A.R. Fersht, Folding of chymotrypsin inhibitor 2. 1. Evidence for a two-state transition, Biochemistry 30 (1991) 10428–10435.
[10] W.A. Klee, F.M. Richards, The reaction of O-methylisourea with bovine pancreatic ribonuclease, J. Biol. Chem. 229 (1957) 489–504.
[11] M. Sela, F.H.J. White, C.B. Anfinsen, Reductive cleavage of disulfide bridges in ribonuclease, Science 125 (1957) 691–692.
[12] F.H.J. White, C.B. Anfinsen, Some relationships of the structure to function in ribonuclease, Ann. N. Y. Acad. Sci. 81 (1959) 515–523.
[13] R. Giri, A. Morrone, C. Travaglini-Allocatelli, P. Jemth, M. Brunori, G. S., Folding pathways of proteins with increasing degree of sequence identities but different structure and function, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 17772–17776.
[14] A. Morrone, M.E. McCully, P.N. Bryan, M. Brunori, V. Daggett, S. Gianni, C. Travaglini-Allocatelli, The denatured state dictates the topology of two proteins with almost identical sequence but different native structure and function, J. Biol. Chem. 286 (2011) 3863–3872.
[15] K.W. Plaxco, M. Gross, Unfolded, yes, but random? Never! Nat. Struct. Biol. 8 (2001) 659–660.
[16] T.L. Religa, J.S. Markson, U. Mayor, S.M. Freund, A.R. Fersht, Solution structure of a protein denatured state and folding intermediate, Nature 437 (2005) 1053–1056.
[17] F. Scaloni, L. Federici, M. Brunori, S. Gianni, Deciphering the folding transition state structure and denatured state properties of nucleophosmin C-terminal domain, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 5447–5452.
[18] F. Scaloni, S. Gianni, L. Federici, B. Falini, M. Brunori, Folding mechanism of the C-terminal domain of nucleophosmin: residual structure in the denatured state and its pathophysiological significance, FASEB J. 23 (2009) 2360–2365.
[19] D. Shortle, M.S. Ackerman, Persistence of native-like topology in a denatured protein in 8 M urea, Science 293 (2001) 487–489.
[20] W.F. van Gunsteren, R. Bürgi, C. Peter, X. Daura, The key to solving the protein-folding problem lies in an accurate description of the denatured state, Angew. Chem. Int. Ed. Eng. 40 (2001) 351–355.
[21] K.B. Wong, J. Clarke, C.J. Bond, J.L. Neira, S.M. Freund, A.R. Fersht, V. Daggett, Towards a complete description of the structural and dynamic properties of the denatured state of barnase and the role of residual structure in folding, J. Mol. Biol. 296 (2000) 1257–1282.
[22] M.J. Parker, J. Spencer, A.R. Clarke, An integrated kinetic analysis of intermediates and transition states in protein folding reactions, J. Mol. Biol. 253 (5) (1995) 771–786.
[23] S. Khorasanizadeh, I.D. Peters, H. Roder, Evidence for a three-state model of protein folding from kinetic analysis of ubiquitin variants with altered core residues, Nat. Struct. Biol. 3 (2) (1996) 193–205.
[24] M.C. Shastry, S.D. Luck, H. Roder, A continuous-flow capillary mixing method to monitor reactions on the microsecond time scale, Biophys. J. 74 (5) (1998) 2714–2721.
[25] T.R. Sosnick, M.D. Shtilerman, L. Mayne, S.W. Englander, Ultrafast signals in protein folding and the polypeptide contracted state, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 8545–8550.
[26] S.W. Englander, Protein folding intermediates and pathways studied by hydrogen exchange, Annu. Rev. Biophys. Biomol. Struct. 29 (2000) 213–238.
[27] S. Akiyama, S. Takahashi, T. Kimura, K. Ishimori, I. Morishima, Y. Nishikawa, T. Fujisawa, Conformational landscape of cytochrome c folding studied by microsecond-resolved small-angle x-ray scattering, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 1329–1334.
[28] H. Roder, Stepwise helix formation and chain compaction during protein folding, Proc. Natl. Acad. Sci. U. S. A. 17 (2004) 1793–1794.
[29] S.J. Hagen, W.A. Eaton, Two-state expansion and collapse of a polypeptide, J. Mol. Biol. 297 (2000) 781–789.
[30] B. Schuler, W.A. Eaton, Protein folding studied by single-molecule FRET, Curr. Opin. Struct. Biol. 18 (2008) 16–26.
[31] B. Schuler, E.A. Lipman, W. Eaton, Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy, Nature 419 (2002) 743–747.
[32] E.V. Kuzmenkina, C.D. Heyes, G.U. Nienhaus, Single-molecule FRET study of denaturant induced unfolding of RNase H, J. Mol. Biol. 357 (2006) 313–324.
[33] K.A. Merchant, R.B. Best, J.M. Louis, I.V. Gopich, W.A. Eaton, Characterizing the unfolded states of proteins using single-molecule FRET spectroscopy and molecular simulations, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1528–1533.
[34] T.Y. Yoo, S.P. Meisburger, J. Hinshaw, L. Pollack, G. Haran, T.R. Sosnick, K. Plaxco, Small-angle X-ray scattering and single-molecule FRET spectroscopy produce highly divergent views of the low-denaturant unfolded state, J. Mol. Biol. 418 (2012) 226–236.

[35] H.M. Watkins, A.J. Simon, T.R. Sosnick, E.A. Lipman, R.P. Hjelm, K.W. Plaxco, Random coil negative control reproduces the discrepancy between scattering and FRET measurements of denatured protein dimensions, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 6631–6636.
[36] D.J. Brockwell, S.E. Radford, Intermediates: ubiquitous species on folding energy landscapes? Curr. Opin. Struct. Biol. 17 (2007) 30–37.
[37] A.P. Capaldi, M.C. Shastry, C. Kleanthous, H. Roder, S.E. Radford, Ultrarapid mixing experiments reveal that Im7 folds via an on-pathway intermediate, Nat. Struct. Biol. 8 (1) (2001) 68–72.
[38] Y. Ivarsson, C. Travaglini-Allocatelli, P. Jemth, F. Malatesta, M. Brunori, S. Gianni, An on-pathway intermediate in the folding of a PDZ domain, J. Biol. Chem. 282 (2007) 8568–8572.
[39] C. Travaglini-Allocatelli, S. Gianni, V. Morea, A. Tramontano, T. Soulimane, M. Brunori, Exploring the cytochrome c folding mechanism: cytochrome c552 from *Thermus thermophilus* folds through an on-pathway intermediate, J. Biol. Chem. 278 (42) (2003) 41136–41140.
[40] P. Jemth, S. Gianni, R. Day, B. Li, C.M. Johnson, V. Daggett, A.R. Fersht, Demonstration of a low-energy on-pathway intermediate in a fast-folding protein by kinetics, protein engineering, and simulation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6450–6455.
[41] A. Matouschek, J.T. Kellis Jr., L. Serrano, M. Bycroft, A.R. Fersht, Transient folding intermediates characterized by protein engineering, Nature 346 (1990) 440–445.
[42] A.R. Fersht, A. Matouschek, L. Serrano, The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding, J. Mol. Biol. 224 (1992) 771–782.
[43] J. Buchner, T. Kiefhaber, Folding pathway enigma, Nature 343 (1990) 601–602.
[44] B.A. Krantz, T.R. Sosnick, Engineered metal binding sites map the heterogeneous folding landscape of a coiled coil, Nat. Struct. Biol. 8 (2001) 1042–1047.
[45] I.E. Sánchez, T. Kiefhaber, Origin of unusual phi-values in protein folding: evidence against specific nucleation sites, J. Mol. Biol. 334 (2003) 1077–1085.
[46] M.A. de los Rios, B.K. Muralidhara, D. Wildes, T.R. Sosnick, S. Marqusee, P. Wittung-Stafshede, K.W. Plaxco, I. Ruczinski, On the precision of experimentally determined protein folding rates and phi-values, Protein Sci. 15 (2006) 553–563.
[47] A.R. Fersht, S. Sato, Phi-value analysis and the nature of protein-folding transition states, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7976–7981.
[48] S. Sato, T.L. Religa, A.R. Fersht, Phi-analysis of the folding of the B domain of protein A using multiple optical probes, J. Mol. Biol. 360 (2006) 850–864.
[49] A.N. Naganathan, V. Muñoz, Insights into protein folding mechanisms from large scale analysis of mutational effects, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 8611–8616.
[50] S. Gianni, P. Jemth, Conserved nucleation sites reinforce the significance of Phi value analysis in protein-folding studies, IUBMB Life 66 (2014) 449–452.
[51] V.I. Abkevich, A.M. Gutin, E.I. Shakhnovich, Specific nucleus as the transition state for protein folding: evidence from the lattice model, Biochemistry 33 (1994) 10026–10036.
[52] A.R. Fersht, Optimization of rates of protein folding: the nucleation-condensation mechanism and its implications, Proc. Natl. Acad. Sci. U. S. A. 21 (1995) 10869–10873.
[53] L.S. Itzhaki, D.E. Otzen, A.R. Fersht, The structure of the transition state for folding of chymotrypsin inhibitor 2 analysed by protein engineering methods: evidence for a nucleation-condensation mechanism for protein folding, J. Mol. Biol. 254 (2) (1995) 260–288.
[54] S. Arrhenius, Uber die Reaktiongeschwindigkeit bei der Inversion von Rohrzucker durch Sauren, Z. Phys. Chem. 4 (1889) 226–248.
[55] Sabelko, J., J. Ervin, and M. Gruebele, Observation of strange kinetics in protein folding. Proc. Natl. Acad. Sci. USA, 199. 96: p. 6031–6036.
[56] F. Huang, L. Ying, A.R. Fersht, Direct observation of barrier-limited folding of BBL by single-molecule fluorescence resonance energy transfer, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 16239–16244.
[57] N. Ferguson, T.D. Sharpe, C.M. Johnson, P.J. Schartau, A.R. Fersht, Structural biology: analysis of 'downhill' protein folding, Nature 445 (2007) 14–15.
[58] F. Huang, S. Sato, T.D. Sharpe, L. Ying, A.R. Fersht, Distinguishing between cooperative and unimodal downhill protein folding, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 123–127.
[59] M.M. Garcia-Mira, M. Sadqi, N. Fischer, J.M. Sanchez-Ruiz, V. Muñoz, Experimental identification of downhill protein folding, Science 298 (2002) 2191–2195.
[60] F.Y. Oliva, V. Munoz, A simple thermodynamic test to discriminate between two-state and downhill folding, J. Am. Chem. Soc. 126 (2004) 8596–8597.
[61] P. Li, F.Y. Oliva, A.N. Naganathan, V. Muñoz, Dynamics of one-state downhill protein folding, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 103–108.
[62] H.S. Chung, J.M. Louis, W.A. Eaton, Experimental determination of upper bound for transition path times in protein folding from single-molecule photon-by-photon trajectories, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11837–11844.
[63] H.S. Chung, K. McHale, J.M. Louis, W.A. Eaton, Single-molecule fluorescence experiments determine protein folding transition path times, Science 335 (2012) 981–984.
[64] H.S. Chung, W.A. Eaton, Single-molecule fluorescence probes dynamics of barrier crossing, Nature 502 (2013) 685–688.
[65] H.S. Chung, S. Piana-Agostinetti, D.E. Shaw, W.A. Eaton, Structural origin of slow diffusion in protein folding, Science 349 (2015) 1504–1510.
[66] K. Lindorff-Larsen, S. Piana, R.O. Dror, D.E. Shaw, How fast-folding proteins fold, Science 334 (2011) 517–520.
[67] M. Shirts, V.S. Pande, COMPUTING: screen savers of the world unite! Science 290 (2000) 1903–1904.
[68] C.D. Snow, H. Nguyen, V.S. Pande, M. Gruebele, Absolute comparison of simulated and experimental protein-folding dynamics, Nature 420 (2002) 102–106.
[69] A. Borgia, K.R. Kemplen, M.B. Borgia, A. Soranno, S. Shammas, B. Wunderlich, D. Nettels, R.B. Best, J. Clarke, B. Schuler, Transient misfolding dominates multidomain protein folding, Nat. Commun. 17 (2015) 8861.
[70] M. Tsytlonok, P.O. Craig, E. Sivertsson, D. Serquera, S. Perrett, R.B. Best, P.G. Wolynes, I. LS., Complex energy landscape of a giant repeat protein, Structure 21 (2013) 1954–1965.
[71] S. Batey, J. Clarke, Apparent cooperativity in the folding of multidomain proteins depends on the relative rates of folding of the constituent domains, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 18113–18118.
[72] J. Danielsson, X. Mu, L. Lang, H. Wang, A. Binolfi, F.X. Theillet, B. Bekei, D.T. Logan, P. Selenko, H. Wennerström, M. Oliveberg, Thermodynamics of protein destabilization in live cells, Proc. Natl. Acad. Sci. U. S. A. 1112 (2015) 12402–12407.
[73] S. Ebbinghaus, A. Dhar, J.D. McDonald, M. Gruebele, Protein folding stability and dynamics imaged in a living cell, Nat. Methods 7 (2010) 319–323.
[74] E.P. O'Brien, J. Christodoulou, M. Vendruscolo, C.M. Dobson, New scenarios of protein folding can occur on the ribosome, J. Am. Chem. Soc. 133 (2011) 513–526.
[75] L.D. Cabrita, C.M. Dobson, C. J., Protein folding on the ribosome, Curr. Opin. Struct. Biol. 20 (2010) 33–45.