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Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales

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Single-molecule spectroscopy has developed into an important method for probing protein structure and dynamics, especially in structurally heterogeneous systems. A broad range of questions in the diversifying field of protein folding have been addressed with single-molecule Förster resonance energy transfer (FRET) and photo-induced electron transfer (PET). Building on more than a decade of rapid method development, these techniques can now be used to investigate a wide span of timescales, an aspect that we focus on in this review. Important current topics range from the structure and dynamics of unfolded and intrinsically disordered proteins, including the coupling of folding and binding, to transition path times, the folding and misfolding of larger proteins, and their interactions with molecular chaperones.

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Introduction

Protein folding research has seen a large degree of diversification in recent years. Even though many of the original questions are far from being fully solved [1], the general focus has shifted away from the classic biochemical studies that had driven the field forward for decades. Instead, we currently observe a development in at least two major directions. One aims at a more detailed physical understanding of the process through an intimate combination of theory, molecular simulations, and advanced experimental methods. Especially the convergence of timescales accessible in recent computer simulations and spectroscopic experiments holds the promise of arriving at unprecedented mechanistic understanding of the folding process in the framework of statistical physics [2–7]. The other direction is based on the increasing realization that the spontaneous self-organization of polypeptide chains into three-dimensional structures has broad functional implications, for example, in the context of intrinsically disordered proteins (IDPs), which appear to be involved in many intracellular interactions and regulatory processes [8,9]. Along similar lines, a more quantitative understanding of the effect of the cellular environment on protein folding mechanisms will be important to bridge the conceptual gap between the traditionally rather separate fields of folding *in vitro* and *in vivo*.

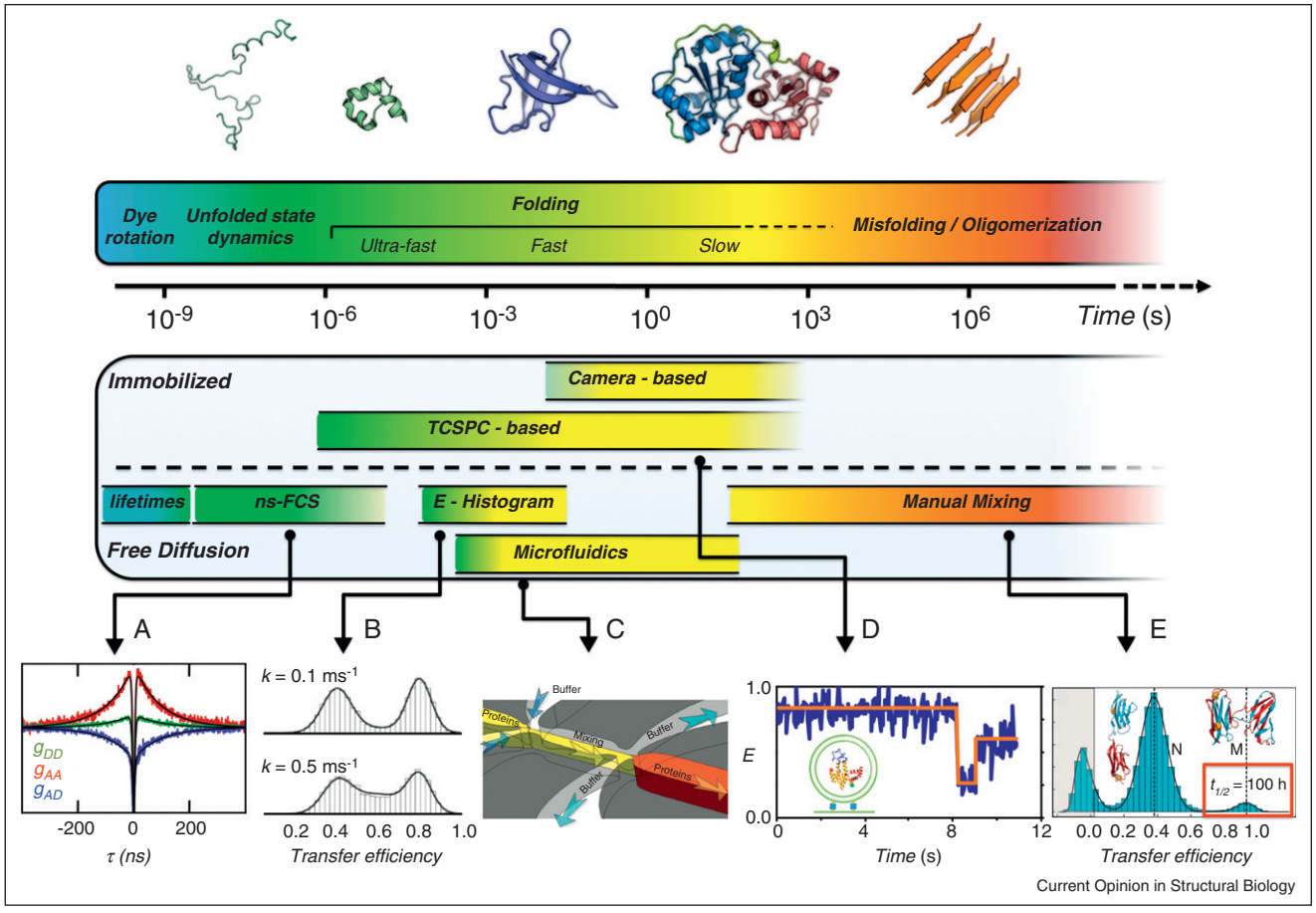
Within the resulting multidisciplinary fields that address questions linked to protein folding, single-molecule methods have started to take an important place [10]. For both research directions outlined above, they can provide valuable and previously unavailable information. Single-molecule experiments are an ideal match for the basic concepts of statistical physics, since they can reveal the distributions and heterogeneity of the structures and dynamics underlying the ensemble average. Intermolecular interactions and the components of the cell add to this heterogeneity, and the ability to probe individual protein molecules in the context of a complex environment, for example, during their interaction with molecular chaperones, or even in an intact cell, further the appeal of single-molecule methods.

Two main types of single-molecule experiments are currently employed in biology: force-probe methods using atomic force microscopy (AFM) or optical tweezers [11], and fluorescence detection, especially in combination with Förster resonance energy transfer (FRET) [12]. AFM and optical tweezers have proven to be a remarkably versatile tool for probing the mechanical stability and folding dynamics of proteins, and the reader is referred to the article by Rief [11] and other recent reviews [10,13]. Here, we focus on the investigation of protein folding with single-molecule fluorescence spectroscopy. In particular, we will review the progress in this area over the past three to four years. During this time, the scope of single-molecule spectroscopy has expanded substantially, both in terms of questions addressed and in terms of timescales accessible. We will summarize this progress with an emphasis on dynamics by going through the relevant range of timescales and relating them to the hierarchy of structure formation in protein folding and binding with examples from the recent literature (Figure 1).

Submicrosecond dynamics: unfolded and intrinsically disordered proteins

Unfolded states have attracted considerable attention, initially because of their general importance as the

Figure 1



Timescales in protein folding accessible with single molecule spectroscopy. Single molecule fluorescence methods, including fluorescence correlation spectroscopy (FCS), cover more than fifteen orders of magnitude in time and allow a wide range of processes relevant for protein folding to be investigated. Essentially all timescales above the lower limit set by the photophysics of the fluorophores can be probed with the available range of experiments and analysis methods on immobilized and/or freely diffusing molecules. The approximate time ranges accessible with different techniques are indicated as horizontal bars. Recent examples for the development and application of these methods are shown at the bottom: **(a)** Autocorrelation and crosscorrelation functions for FRET-labeled unfolded cyclophilin at 1.5 M GdmCl obtained with ns-FCS, from which the chain reconfiguration dynamics can be determined [25•,28,52]. **(b)** Interconversion times between species can be obtained from the analysis of transfer efficiency histograms. The solid lines are fits to simulated data according to the theory of Gopich and Szabo [30]. (Figure adapted from [30].) **(c)** Schematic of a microfluidic mixing device with a dead time of 200 μs for single-molecule detection designed by Gambin *et al.* [81•]. (Figure taken from [81•].) **(d)** Example of a FRET efficiency trajectory of immobilized adenylate kinase, a multi-domain protein whose folding dynamics were investigated by Pirchi *et al.* [73•]. (Data from [73•].) **(e)** Transfer efficiency histogram of a refolded I27 tandem repeat with 5% misfolded molecules at high transfer efficiencies (red box) that convert back to the native species with a half-life of about one week [98•]. (Figure adapted from [98•].)

starting point of the protein folding reaction [14], later in the context of the 'speed limit' of folding: structure formation cannot be faster than the diffusive encounter of the parts of the polypeptide chain forming interactions [15–17]. Renewed interest in the properties of unfolded proteins has come with the identification of intrinsic disorder in large parts of eukaryotic proteomes and the investigation of its functional importance [8,9]. Single-molecule spectroscopy is ideally suited for probing such structurally heterogeneous and dynamic systems [18,19] and for complementing the information available from methods such as NMR [8,20].

Single-molecule FRET, which allows distances and distance dynamics to be probed in a range from about 2 nm to 10 nm (Box 1), has been used very successfully to investigate unfolded proteins [21,22] and IDPs [18,23]. Advances in methodology, in particular the use of fluorescence lifetimes and anisotropies in addition to fluorescence count rates from donor and acceptor [24,25•], rigorous data analysis, especially based on the development of comprehensive theoretical concepts [24,26–28,29•,30,31•,32,33] now enables distances [24], distance distributions [34,35], and interconversion dynamics [36•,37•] to be obtained very accurately [21]. An