Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Structural Biology

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

Benjamin Schuler and Hagen Hofmann

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.

Address
University of Zurich, Department of Biochemistry, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Corresponding author: Schuler, Benjamin (schuler@bioc.uzh.ch)

Current Opinion in Structural Biology 2013, **23**:xx–yy

This review comes from a themed issue on **Folding and binding**

Edited by **Jayant Udgaonkar** and **Susan Marqusee**

http://dx.doi.org/10.1016/j.sbi.2012.10.008

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.

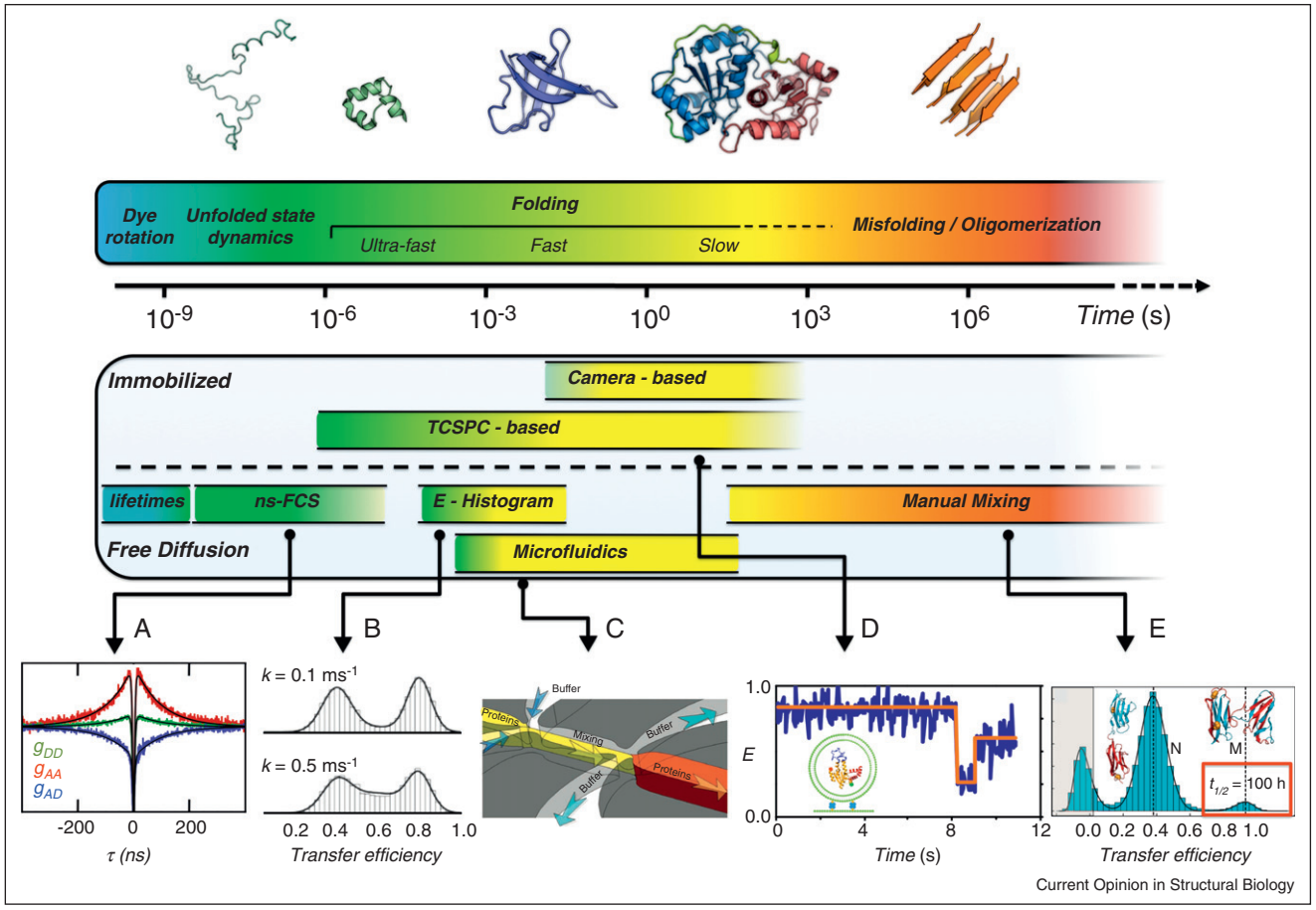
Two main types of single-molecule experiments are currently employed in biology: force-probe methods using atomic force microscopy (AFM) or optical tweezers [11], and fluorescence detection, especially in combination with Förster resonance energy transfer (FRET) [12]. AFM and optical tweezers have proven 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 in this issue [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

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

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].

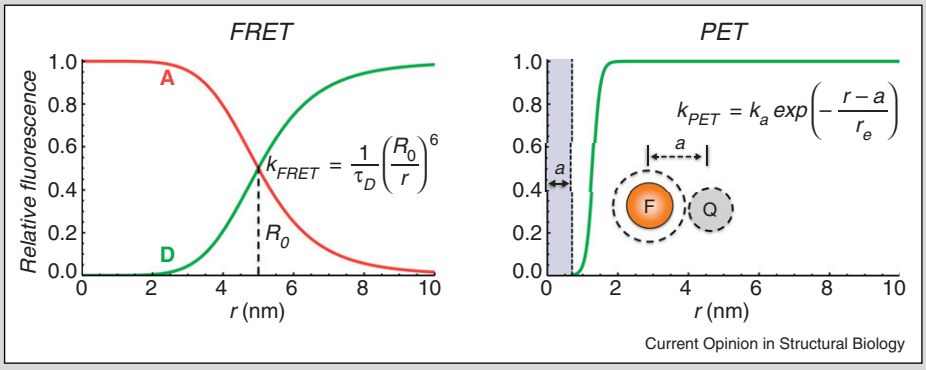
Single-molecule FRET, which allows distances and distance dynamics to be probed in a range from about 2 nm to 10 nm (Box 1), has been used very successfully to investigate unfolded proteins [21,22] and IDPs [18,23]. Advances in methodology, in particular the use of fluorescence lifetimes and anisotropies 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

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

Box 1 Comparison of FRET and PET

In its classical description, Förster resonance energy transfer (FRET) originates from the resonance of the transition dipole moments of donor (D) and acceptor (A) fluorophores. The rate constant of the resulting energy transfer (k_{FRET}) from D to A depends on the inverse 6th power of the D–A distance (r), the fluorescence lifetime of the donor (τ_D), and the characteristic distance R_0 that can be calculated from the spectroscopic properties of D and A. The typical values of R_0 make single-molecule FRET suitable for obtaining distance information in the range of ~2 nm to ~10 nm. By contrast, photo-induced electron transfer (PET) requires much closer contact between fluorophore (F) and quencher (Q, typically tryptophan). The rate constant of quenching (k_{PET}) depends on the contact radius (a), a characteristic distance (r_e) in the subnanometer range, and the quenching rate constant at the distance of closest approach (k_a). The on/off behavior resulting from the steep distance dependence of the fluorescence emission in PET can in principle be used to probe any process that leads to a change in quenching dynamics, but it does not afford direct distance information. As an example, both PET and FRET, when combined with ns-FCS, can provide information on the dynamics of unfolded and disordered proteins in the nanosecond to microsecond time regime. FRET-FCS can be used to determine the reconfiguration time of the chain; in addition, the distance or distance distribution between D and A can be obtained from the analysis of transfer efficiencies and/or fluorescence lifetimes. PET-FCS allows the determination of loop-closure rates between two points within a polypeptide chain. The complementarity of the two methods is expected to be increasingly useful for probing biomolecular dynamics over a wide range of distances and times (Figure I).

Figure I



important strength of single-molecule FRET is the separation of folded and unfolded subpopulations [38,39]. As a result, the properties of the unfolded state can be quantified even under conditions where the majority of molecules are folded and would thus dominate the signal in an ensemble experiment. This separation of subpopulations led to the identification of the continuous compaction, or collapse, of the unfolded state with decreasing concentration of denaturant [39], a rather generic behavior [21,22] (and references therein) that reflects the change in solvent quality for the polypeptide chain [14,40,41]. Most recently, this approach was used to quantify the effect of sequence composition on the length scaling of unfolded proteins and IDPs within the framework of polymer theory [41].

This type of experiments can also be used to investigate the effect of other changes in conditions on unfolded proteins. In response to an increase in temperature, for example, Nettels *et al.* [42] observed a collapse of an unfolded cold shock protein and the intrinsically disordered protein prothymosin α, suggesting an increase in intramolecular interactions upon heating. Molecular dynamics simulations indicated an important role of solvation and the possible involvement of secondary structure formation [42,43] in this process. For IDPs, which frequently contain a large fraction of charged amino acids, a contribution of particular importance is the interaction between the charges in the polypeptide chain. Single-molecule FRET experiments in combination with an analysis based on polyampholyte theory showed that charge repulsion can in fact dominate the chain dimensions of IDPs and lead to a pronounced chain expansion at low ionic strength [44,45], in agreement with results from simulations [46]. Surprisingly, however, attraction between opposite charges within polypeptide chains with low net charge can lead to an additional compaction, as predicted by polyampholyte theory [44]. Structural and dynamic properties of more complex IDPs are also starting to be investigated with single-molecule spectroscopy [47–49].

The quantitative description of experimentally derived distance distributions and intramolecular interactions in terms of polymer models has been very successful [35,44,50,51], and the corresponding distance information provides the first important piece of information for understanding the dynamics of unfolded proteins and IDPs. The second piece of information, the timescale of chain dynamics, can be obtained from nanosecond correlation spectroscopy (ns-FCS) [28,52,53] (Figure 1a), which can be readily implemented in confocal instruments with the latest generation of counting electronics [54]. In ns-FCS experiments based on FRET, the long-range distance fluctuations between the donor and the acceptor fluorophore report on the characteristic timescale of

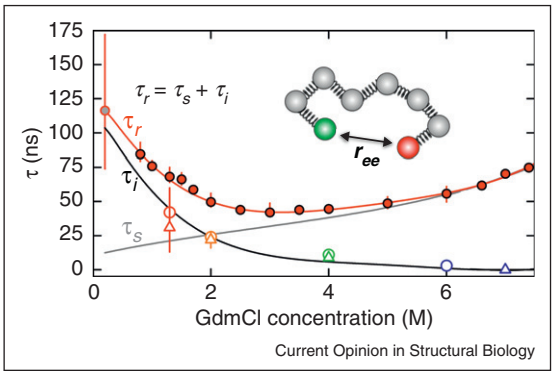
Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

interconversion between different configurations of the polypeptide chain. The resulting relaxation times are in the range of tens of nanoseconds to ~200 ns for the segment lengths from ~30 to ~200 amino acid residues investigated to date [25•,28,52,53]. These times are remarkably close to the reconfiguration times expected for ideal polymers [55], indicating that the underlying dynamics approach chain diffusion in the absence of persistent interactions within the polypeptide.

However, at low denaturant concentrations, the reconfiguration time can increase substantially above the values expected for an ideal chain [25•,52]. This behavior had been ascribed to 'internal friction' caused by dissipative mechanisms within the polypeptide, such as dihedral angle rotations or transient side chain or backbone interactions [52], but it had eluded a quantitative description. The recent work of Soranno *et al.* [25•] (Figure 2) illustrates the use of polymer-physical principles in combination with single-molecule FRET and ns-FCS for quantifying internal friction in unfolded proteins and IDPs. Polymer dynamics models, such as the Rouse model with internal friction [56], show that the reconfiguration time, τ_r, of a polymer can be decomposed into a sum of two relaxation times, that of the ideal chain, τ_0, and a contribution from internal friction, τ_i, with $\tau_r = \tau_0 + \tau_i$. Based on this relationship and solvent-viscosity-dependent and position-dependent measurements of unfolded state dynamics, Soranno *et al.* [25•] showed for a number of proteins that internal friction is close to zero at very high concentrations of denaturant, where the chains are very expanded and thus behave similar to an ideal polymer. For very compact chains, however, τ_i can dominate the chain dynamics and increase τ_r up to an order of magnitude above the value expected for an ideal chain. Highly charged IDPs do not exhibit this compaction but are expanded owing to charge repulsion [44,46], which reduces internal friction and may affect the dynamics of their interactions with cellular binding partners [25•,57]. In summary, single-molecule FRET in combination with ns-FCS provides an opportunity to quantify the properties of unfolded and disordered proteins even on submicrosecond timescales and rationalize them in terms of polymer-physical concepts. Interestingly, the estimate of the pre-exponential factor or 'speed limit' of the folding reaction based on these results and a simple Kramers model is in the range of 0.5 μs [52], which is surprisingly close to the folding times of the fastest folding proteins [16] and their transition path times [58••], suggesting that changes in unfolded state dynamics can affect folding rates [59] (see also *Microsecond dynamics: barriers, transition paths, and folding at the speed limit*).

A single-molecule method complementary to FRET that has started to provide important dynamic information on the submicrosecond timescale is photo-induced electron transfer (PET) combined with ns-FCS, pioneered by Sauer and colleagues [60]. In this case, static quenching of a fluorophore by tryptophan is exploited for obtaining dynamics based on contact formation within proteins or peptides, conceptually related to some ensemble quenching methods [15,17,61] and complementary to the long-range dynamics accessible with FRET (Box 1). Recent applications include studies of model peptides [60,62], for example, in the context of molecular crowding [63], and fast-folding proteins [64,65•]. Neuweiler *et al.* [65•] used PET to monitor the 10 μs-folding time and the submicrosecond contact rate in the denatured state of the small protein BBL, including the presence of internal friction. The same method was applied to investigate the surprisingly slow dynamics in a trapped intermediate of engrailed homeodomain [64], which the authors assigned to the presence of nonnative interactions that lead to interconversion on a timescale similar to that of the overall folding process.

Figure 2



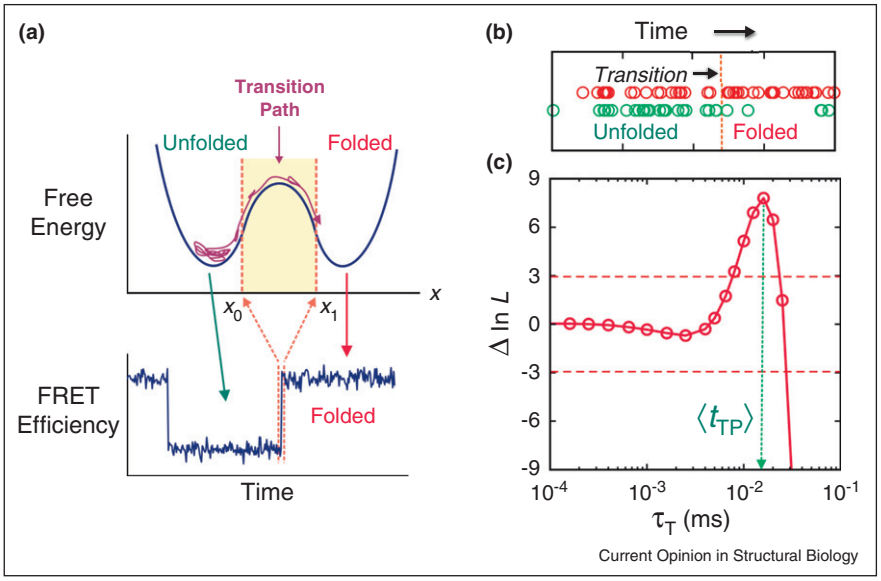
Internal friction in an unfolded protein quantified in a combination of single-molecule FRET and ns-FCS with concepts from polymer dynamics [25•]. The experimentally determined GdmCl dependence of the end-to-end reconfiguration time, τ_r, of unfolded cold shock protein obtained in equilibrium measurements (filled red circles) and from microfluidic mixing (gray filled circle) is shown with a polynomial fit used for interpolation (red line). The gray line shows the reconfiguration time expected for a Rouse model in the absence of internal friction (τ_s). The characteristic timescale associated with internal friction (τ_i) calculated from $\tau_r = \tau_s + \tau_i$ is shown as a black line. The independently determined values of τ_i are shown as open circles and triangles, respectively, for comparison. Inset: Schematic of a polymer in the Rouse model, represented as beads connected by harmonic potentials indicated as springs. The end-to-end distance (r_{ee}) and its fluctuations are probed by FRET between donor (green) and acceptor (red). (Figure adapted from [25•].)

Microsecond dynamics: barriers, transition paths, and folding at the speed limit

Folding dynamics on the microsecond timescale have been a particularly attractive research topic in recent years. Many proteins that fold in microseconds still exhibit the key structural properties of folded proteins, with specific tertiary structure and a hydrophobic core [42], and their kinetics are starting to become accessible to all-atom

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

Figure 3



Experimental determination of the folding transition path time for a two-state protein [58••]. (**a**, top) Schematic of the kinetics of protein folding represented by diffusion on a one-dimensional free-energy surface with an order parameter (x) as a reaction coordinate. The unfolded molecule spends the vast majority of time visiting a large number of conformations in the free energy well of the unfolded state. A transition path is the part of the trajectory that crosses the point x_0 on the reaction coordinate and reaches x_1 on the other side of the barrier without recrossing x_0. The duration of this process is the transition path time. (**a**, bottom) Sketch of a corresponding FRET efficiency trajectory showing transitions between the folded state (high FRET efficiency) and the unfolded state (low FRET efficiency). The duration of the jump in the trajectory corresponds to the transition path time. **(b)** Example of donor (green) and acceptor (red) photons recorded from an immobilized WW-domain molecule, showing a transition from unfolded to folded. **(c)** The difference between the log likelihoods of a two-state model with a finite transition path time and a two-state model with an instantaneous transition, $\Delta \ln L = \ln L(\tau_T) - \ln L(0)$, is plotted as a function of the transition path time τ_T. The value for τ_T at the maximum of $\Delta \ln L$ identifies the most likely average transition path time, $\langle t_{TP} \rangle$. (Figure adapted from [58••].)

simulations [4,6]. The convergence between experiments and simulations on this timescale has thus become a very promising avenue towards a detailed understanding of protein folding mechanisms. The use of single-molecule fluorescence is challenging on the microsecond timescale and below, because the maximum average photon count rate from an individual molecule is only about 10^5 Hz to 10^6 Hz owing to the limited photon collection efficiencies of current instruments and the photophysical properties of the fluorophores. However, the broad distribution of interphoton times results in a finite probability of observing shorter interphoton times, and rapid correlation methods as described in *Submicrosecond dynamics: unfolded and intrinsically disordered proteins* allow access to this time range, both in FRET and PET experiments [36•,37•,60,64,65•,66].

A different approach to probing the microsecond range is illustrated by the remarkable recent use of single-molecule FRET for measuring transition path times of protein folding (Figure 3). While the chemical kinetics of protein folding contain the information on the *frequency* of the folding and unfolding transitions, the information on the *mechanism*, that is, the sequence of microscopic events that take the protein from the unfolded to the folded state, is contained in the actual process of barrier crossing. An ultimate goal of single-molecule experiments will be to resolve these transitions [21,67], which is currently only possible in computer simulations. The distribution of transition paths may in fact only be experimentally accessible with single-molecule observations. A first important step towards this goal is to determine the time it takes a molecule to cross the barrier. Building on earlier work [68], Chung *et al.* [58••] have recently succeeded in measuring the average transition path time for the FBP28 WW domain (folding time ~100 μs) and an upper bound for protein GB1 (folding time ~1 s) by analyzing the photon statistics of a very large number of fluorescence trajectories from immobilized molecules with a maximum likelihood method developed by Gopich and Szabo [29•]. In spite of the very different folding times of the two proteins, their transition path times are remarkably similar, with ~2 μs for the WW domain and <10 μs for GB1 [58••]. From all-atom explicit solvent simulations on the similar FiP35 WW domain, Shaw *et al.* [4] found a very similar average transition path time of ~1.5 μs (after correcting the observed 0.5 μs for the 3-fold smaller viscosity of the water model used in the simulations), which is also expected to be closely related to the ~1.5 μs 'molecular phase' observed in ensemble

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

laser temperature jump experiments on FiP35 WW domain [69]. These times are comparable to the protein folding 'speed limit' in the range of ~1 μs previously estimated from experimental and theoretical approaches [16], and remarkably close to the unfolded state dynamics from nsFCS [25•,52,65•] (see *Submicrosecond dynamics: unfolded and intrinsically disordered proteins*). It will also be interesting to see whether a correlation exists between transition path times and the presence of internal friction at the transition state, for example, for the spectrin domains, some of which were recently found to exhibit unusually high internal friction in their folding reactions [70].

A key benefit of single-molecule experiments is the possibility to extract dynamic information even from equilibrium measurements, which avoids the necessity for a synchronization of the system by perturbation methods, as frequently employed in ensemble experiments. For protein folding, equilibrium dynamics have been obtained, for example, from correlation functions [25•,52,65•,66,71] (Figure 1a), the analysis of broadening and exchange between subpopulations in FRET efficiency histograms [30,36•,37•,39] (Figure 1b), and from fluorescence trajectories of immobilized molecules [36•,58••,66–68,72,73•,74] (Figure 1d). In this way, both the equilibrium distributions and the kinetics can sometimes be obtained from the same measurement.

A recent example for which this possibility has been of great interest is the distinction of two-state and downhill (or 'one-state') protein folding. In single-molecule FRET experiments on freely diffusing molecules, two-state behavior will result in two separate subpopulations in the FRET efficiency histograms if the interconversion kinetics are slower than the observation time [27], which is determined by the ~1 ms diffusion time of the molecules through the confocal volume, and by the photon count rate during this time, which limits the minimal binning time that can be used for the analysis. In the case of much faster exchange or for a one-state scenario, a single peak that shifts continuously with denaturant concentration is expected. For BBL, a small protein previously suggested to be a one-state folder by Muñoz and colleagues [75], Huang *et al.* [76] reported a separation of folded and unfolded subpopulations at an observation time of 50 μs, and, from ensemble temperature-jump experiments, a relaxation time for their interconversion at 4 M guanidinium chloride (GdmCl) of ~340 μs, suggesting barrier-limited folding kinetics. Recently, this finding was challenged by Liu *et al.* [77], who reported their single-molecule FRET experiments on a slightly different variant of BBL to be incompatible with two-state behavior. They obtained a relaxation time of ~200 μs from a correlation analysis of their single-molecule data at 6.1 M urea and ensemble temperature-jump experiments at 2.2 M GdmCl. To clarify the origin of this discrepancy, further experiments will be required, ideally with instrumentation that enables single-photon counting, to obtain additional observables, such as fluorescence lifetimes and anisotropies, and to allow a more detailed analysis of the photon statistics.

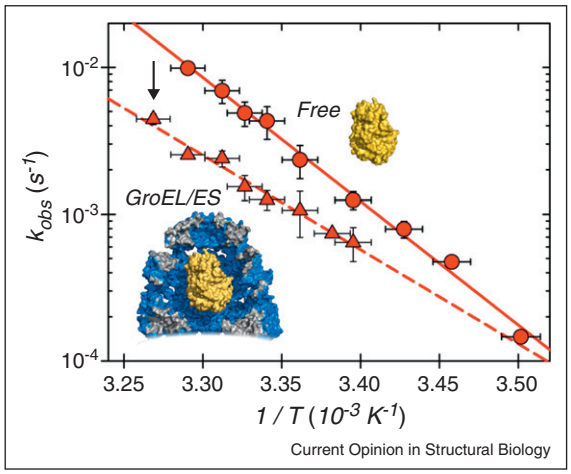
Millisecond dynamics: microfluidics coming of age

Even though it is often possible to obtain information on kinetics from equilibrium single-molecule experiments, in many cases it is still required to probe nonequilibrium dynamics, especially if the reaction of interest is essentially irreversible during the observation time accessible at equilibrium. A method that lends itself very well to the combination with single-molecule detection optics is microfluidic mixing, and since the first application to protein folding [78], several different implementations have been reported [79,80,81•,82]. The basic idea of such devices is to mix solutions in continuous laminar flow by reducing the dimensions such that the components of the solutions that are combined exchange very quickly, solely by diffusion [83]. Since the typical length scale over which small molecules such as denaturants diffuse in a millisecond is about a micrometer, structures in this size range are required to obtain dead times comparable to established methods based on turbulent mixing, such as stopped-flow. After mixing, the confocal observation volume is placed at different points in the observation channel, corresponding to different times after mixing. In a recent mixer design (Figure 1c), Gambin *et al.* [81•] achieved a dead time of ~200 μs, very close to the limits of time resolution dictated by the minimum residence time that is required to observe a sufficient number of photons from a single molecule flowing through the confocal volume. They used the device to investigate the kinetics of the structural transitions of the IDP α-synuclein upon interaction with sodium dodecyl sulfate (SDS) and were able to resolve the formation of an intermediate that they assigned to a helix–turn–helix structure preceding the final extended helix bound to micelles [81•].

A key step towards the usability required for a broader application of microfluidic mixing in protein folding and binding studies has been the utilization of replica-molding in elastomers such as polydimethylsiloxane (PDMS)[84], which greatly simplifies the production of the devices from a microfabricated mold in large numbers and increases reproducibility [80,85]. A mixer design optimized along these lines for single-molecule fluorescence experiments [80] has recently been employed to investigate internal friction in unfolded proteins [25•] and protein folding inside the GroEL/ES chaperonin cavity [86•]. Soranno *et al.* [25•] used the device in combination with ns-FCS to quantify internal friction in unfolded proteins (see *Submicrosecond dynamics: unfolded and intrinsically disordered proteins*). The

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

Figure 4



Direct comparison of folding dynamics outside and inside the GroEL/ES chaperonin cavity from single molecule fluorescence spectroscopy [86•]. The rate constants of folding of the C-terminal domain of rhodanese inside the single-ring GroEL/ES cavity (triangles) are reduced compared to folding free in solution (circles) over a wide temperature range, suggesting that interactions between denatured rhodanese and the cavity walls and/or internal friction can slow down folding inside the chaperonin cage. Insets: Schematic surface representations of native rhodanese (top) and native rhodanese inside the GroEL-GroES cavity.

microfluidic mixer allowed the unfolded state of a protein to be transiently populated at low denaturant concentrations (where it is folded at equilibrium); in this way, the reconfiguration time could be determined under conditions where the unfolded state is most compact (Figure 2). Owing to the continuous-flow mixing employed, even correlation experiments with long acquisition times can thus be performed on such non-equilibrium states. Microfluidic mixing also provides new opportunities for studying protein folding and binding in systems involving multiple components and greater structural complexity. Hofmann *et al.* [86•] used it for probing the folding of the chaperone substrate protein rhodanese upon encapsulation in the GroEL/ES chaperonin cavity. The absence of obvious changes in the transfer efficiency histograms of FRET-labeled rhodanese bound to GroEL in the first few seconds after binding of ATP and the co-chaperone GroES suggested that the forced unfolding proposed for other substrates [87,88] does not occur in the case of rhodanese.

Seconds, minutes, and beyond: fluorescence trajectories and free diffusion

Observing one individual molecule at equilibrium for more than a few milliseconds usually requires immobilization. In contrast to experiments on freely diffusing molecules, where confocal detection is used predominantly, two detection schemes are commonly employed for immobilized molecules. Confocal detection in combination with avalanche photodiodes has the advantage of subnanosecond time resolution, and provides fluorescence lifetime information and anisotropy decays if combined with time-correlated single photon counting (TCSPC) [24], but the molecules need to be interrogated sequentially. For monitoring slower dynamics of many immobilized molecules in parallel in a larger field of view, sensitive CCD cameras with their millisecond frame rates, combined with total internal reflection fluorescence (TIRF) are becoming increasingly popular. Building on earlier studies [72,74,89], many of the original limitations of immobilization experiments have been overcome. An important factor is the use of photoprotectants [42,90–92] to minimize the photochemistry that leads to blinking (which can easily be misinterpreted as conformational dynamics [93]), and to reduce bleaching, which limits the length of fluorescence trajectories from single molecules.

Two interesting examples of equilibrium folding dynamics from single-molecule FRET experiments on the timescale of seconds are recent reports on immobilized adenylate kinase [73•] and on several IDPs [94•]. Based on a Markov-state analysis of thousands of fluorescence trajectories from confocal measurements of the three-domain protein adenylate kinase encapsulated in lipid vesicles (Figure 1d), Pirchi *et al.* [73•] reported evidence for about six distinct interconverting states whose connectivity depends on the denaturant concentration. This work illustrates that even the folding dynamics of larger multi-domain proteins are starting to become accessible with single-molecule experiments. Using a camera-based approach, Choi *et al.* [94•] made a surprising observation on some proteins previously categorized as intrinsically disordered. For three of them, the transfer efficiency values were observed to be constant on the millisecond integration time of the camera, as expected from the rapid conformational dynamics expected for IDPs (see *Submicrosecond dynamics: unfolded and intrinsically disordered proteins*). But neuroligin and the NMDAR-2B glutamate receptor exhibited 'conformational switching' on the time scale of seconds, indicating the presence of unexpectedly large free energy barriers. In both examples [73•,94•], the structural identity of the transient states is currently unclear, but future experiments with multiple dye pairs, complementary methods, or the combination with simulations might facilitate a structural assignment.

While immobilization is required for observing long fluorescence trajectories from individual molecules, the temporal evolution of population distributions can also be monitored with confocal experiments on freely diffusing molecules, which can be of interest especially for non-equilibrium experiments on very slow processes. Simple manual mixing can be used to extend the observation time to any range that sample stability permits. By

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

combining microfluidic and manual mixing experiments, Hofmann *et al.* [86•] were able to monitor the folding of rhodanese, an aggregation-prone chaperone substrate, in the GroEL/ES chaperonin cavity from the encapsulation (see *Millisecond dynamics: microfluidics coming of age*) to the completion of the folding reaction over about one hour. Since substrate protein aggregation is negligible at the picomolar protein concentrations in these experiments, the authors could exclude the contribution of this side reaction and thus rigorously compare the folding rates of rhodanese in the cavity to those free in solution, outside the chaperone. The surprising observation was that the C-terminal domain of rhodanese folds up to a factor of eight more slowly inside the chaperonin cavity than in free solution (Figure 4), while the N-terminal domain and the sequence of folding events are unaffected. This reduction in folding rate was suggested to be a result of interactions with the cavity walls or confinement-induced internal friction, as previously proposed based on simulations [95–97].

A case of an extremely slow process monitored by single-molecule FRET was reported by Borgia *et al.* in their studies of misfolding in tandem repeats of Ig-like domains from titin [98•] (Figure 1e). They demonstrated that 5% of I27-I27 repeats form a misfolded structure upon dilution from denaturant. In the misfolded molecules, the swapped arrangement of the terminal β-strands of the two domains is identical to that within a single domain in the properly folded structure, which explains the mechanical stability of the misfolded state [99]. Interestingly, the probability of misfolding is lower for tandem repeats with lower sequence similarity, supporting the hypothesis that low sequence similarity of neighboring domains in multi-domain proteins is a result of evolutionary pressure to avoid misfolding [100]. The clear separation of folded, unfolded, and misfolded subpopulations also allowed the kinetics of all subpopulations to be monitored as a function of denaturant. Based on molecular simulations and the slow unfolding kinetics of the misfolded state, the authors concluded that in the misfolded state, two stably folded domains are formed. However, over longer times, the misfolded subpopulation spontaneously converts back to the folded state, indicating that misfolding is a result of kinetic partitioning, but that the natively folded structure corresponds to the free energy minimum. In the absence of denaturant, the conversion from the misfolded to the folded state exhibits a relaxation time of about one week [98•]. This example also illustrates the possibility to obtain structural and kinetic information even on very low populated states that would be inaccessible with ensemble experiments.

Conclusions

Single-molecule spectroscopy has progressed remarkably over the past few years, and we have seen a transition from feasibility studies and proof-of-principle experiments to an important tool for gaining conceptual insight in protein structure and dynamics that would be difficult to obtain otherwise. Particularly remarkable is the broad range of timescales now accessible with single-molecule spectroscopy. Already the examples reviewed here cover 13 orders of magnitude, essentially all timescales relevant for protein folding dynamics (Figure 1).

In spite of these advances, single-molecule methods are still developing rapidly, and many of the experimental techniques and analysis methods introduced only a few years ago are now an established part of the state-of-the-art toolbox. Aside from further developments in the directions mentioned above, we expect a particularly important impact on protein folding and binding from several newly emerging methods. Three-color FRET is clearly one of them: monitoring up to three distances at a time allows correlations to be established between conformational changes in different parts of a protein or between folding and binding events in IDPs [101]. Related goals might be attainable with a direct combination of FRET and PET. The continued development of methods to analyze single molecule experiments and to model photon statistics [24,26–33,62,102,103] will play an important role for dissecting systems of increasing complexity. The biochemically very demanding specific labeling of proteins with three chromophores will benefit from improved labeling and chromophore incorporation strategies [104,105].

Technical developments that are expected to have a major impact (in particular on experiments based on fluorescence trajectory analysis) include zero-mode waveguide arrays for increased throughput [106], single photon avalanche diode arrays [107] for the combined advantages of area detectors with time-correlated single photon counting and full correlation analysis, and methods that allow individual molecules to be monitored for extended times without requiring immobilization [103], to name but a few. A key requirement for improving the time resolution of single-molecule fluorescence trajectories will be to increase the photon emission rate by further optimization of photoprotective additives and related methods [92].

Advances of this kind will help to further expand the accessible timescales and the scope of single-molecule spectroscopy for addressing the diverse range of questions related to protein folding and dynamics. For all levels of complexity, from fundamental aspects such as unfolded state/IDP dynamics [23,25•] and transition paths [58••] to the role of misfolding and alternative native structures [98•,108], molecular chaperones [86•,87], and experiments *in vivo* [109•], a quantitative approach will be essential for bridging the divide between these areas, often in combination with simulation and theory. Even

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

though current evidence indicates no fundamental differences between folding and binding processes *in vitro* and *in vivo* [110,111], even moderate modulations of rates and equilibria might be relevant *in vivo*, and understanding the influence of cellular factors in more mechanistic detail will certainly be essential. The potential of single-molecule spectroscopy for investigating heterogeneous systems in the context of interactions with a broad range of environmental factors thus provides many opportunities for reaching a more comprehensive picture of the mechanisms and functional roles of protein folding processes.

Acknowledgements

We thank Gilad Haran for providing the data shown in Figure 1d. Our work has been supported by the Swiss National Science Foundation, the National Center of Competence in Research for Structural Biology, a Starting Investigator Grant of the European Research Council, and the Human Frontier Science Program.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Sosnick TR, Barrick D: **The folding of single domain proteins–have we reached a consensus?** *Curr Opin Struct Biol* 2011, **21**:12-24.

2. Onuchic JN, Wolynes PG: **Theory of protein folding**. *Curr Opin Struct Biol* 2004, **14**:70-75.

3. Dill KA, Ozkan SB, Shell MS, Weikl TR: **The protein folding problem**. *Annu Rev Biophys* 2008, **37**:289-316.

4. Shaw DE, Maragakis P, Lindorff-Larsen K, Piana S, Dror RO, Eastwood MP, Bank JA, Jumper JM, Salmon JK, Shan Y *et al.*: **Atomic-level characterization of the structural dynamics of proteins**. *Science* 2010, **330**:341-346.

5. Thirumalai D, O'Brien EP, Morrison G, Hyeon C: **Theoretical perspectives on protein folding**. *Annu Rev Biophys* 2010, **39**:159-183.

6. Freddolino PL, Harrison CB, Liu Y, Schulten K: **Challenges in protein folding simulations: timescale, representation, and analysis**. *Nat Phys* 2010, **6**:751-758.

7. Bowman GR, Voelz VA, Pande VS: **Taming the complexity of protein folding**. *Curr Opin Struct Biol* 2011, **21**:4-11.

8. Dyson HJ, Wright PE: **Intrinsically unstructured proteins and their functions**. *Nat Rev Mol Cell Biol* 2005, **6**:197-208.

9. Uversky VN, Oldfield CJ, Dunker AK: **Intrinsically disordered proteins in human diseases: introducing the D2 concept**. *Annu Rev Biophys* 2008, **37**:215-246.

10. Borgia A, Williams PM, Clarke J: **Single-molecule studies of protein folding**. *Annu Rev Biochem* 2008, **77**:101-125.

11. Rief M: **Force as a Single Molecule Probe of Multidimensional Protein Energy Landscapes**. *Curr. Opin. Struct. Biol*, http://dx.doi.org/10.1016/j.sbi.2012.11.007.

12. Selvin PR, Ha T: *Single-Molecule Techniques: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press; 2008.

13. Hummer G, Szabo A: **Free energy surfaces from single-molecule force spectroscopy**. *Acc Chem Res* 2005, **38**:504-513.

14. Dill KA, Shortle D: **Denatured states of proteins**. *Annu Rev Biochem* 1991, **60**:795-825.

15. Hagen SJ, Hofrichter J, Szabo A, Eaton WA: **Diffusion-limited contact formation in unfolded cytochrome c: estimating the maximum rate of protein folding**. *Proc Natl Acad Sci USA* 1996, **93**:11615-11617.

16. Kubelka J, Hofrichter J, Eaton WA: **The protein folding 'speed limit'**. *Curr Opin Struct Biol* 2004, **14**:76-88.

17. Bieri O, Wirz J, Hellrung B, Schutkowski M, Drewello M, Kiefhaber T: **The speed limit for protein folding measured by triplet-triplet energy transfer**. *Proc Natl Acad Sci USA* 1999, **96**:9597-9601.

18. Schuler B, Müller-Späth S, Soranno A, Nettels D: **Application of Confocal Single-Molecule FRET to Intrinsically Disordered Proteins**. In *Intrinsically Disordered Protein Analysis: Volume 2, Methods and Experimental Tools*, vol 896. Edited by Uversky VNaD AK. Springer; 2012:21-45.

19. Ferreon ACM, Gambin Y, Lemke EA, Deniz AA: **Interplay of alpha-synuclein binding and conformational switching probed by single-molecule fluorescence**. *Proc Natl Acad Sci USA* 2009, **106**:5645-5650.

20. Schneider R, Huang JR, Yao M, Communie G, Ozenne V, Mollica L, Salmon L, Jensen MR, Blackledge M: **Towards a robust description of intrinsic protein disorder using nuclear magnetic resonance spectroscopy**. *Mol Biosyst* 2012, **8**:58-68.

21. Schuler B, Eaton WA: **Protein folding studied by single-molecule FRET**. *Curr Opin Struct Biol* 2008, **18**:16-26.

22. Haran G: **How, when and why proteins collapse: the relation to folding**. *Curr Opin Struct Biol* 2012, **22**:14-20.

23. Ferreon AC, Moran CR, Gambin Y, Deniz AA: **Single-molecule fluorescence studies of intrinsically disordered proteins**. *Methods Enzymol* 2010, **472**:179-204.

24. Sisamakis E, Valeri A, Kalinin S, Rothwell PJ, Seidel CAM: **Accurate Single-Molecule FRET Studies Using Multiparameter Fluorescence Detection**. *Methods Enzymol* 2010, **475**:455-514.

25. • Soranno A, Buchli B, Nettels D, Müller-Späth S, Cheng RR, Pfeil SH, Hoffmann A, Lipman EA, Makarov DE, Schuler B: **Quantifying internal friction in unfolded and intrinsically disordered proteins with single molecule spectroscopy**. *Proc Natl Acad Sci USA* 2012 http://dx.doi.org/10.1073/pnas.1117368109. early edition.
By combining single-molecule FRET, ns-FRET-FCS, and concepts from polymer physics, the contribution of internal friction to the dynamics of unfolded and disordered proteins could be quantified for the first time. Surprisingly, internal friction exceeds solvent friction by almost one order of magnitude in the absence of denaturant, suggesting that internal friction in the unfolded state may affect the folding rates of ultra-fast-folding proteins.

26. Gopich IV, Szabo A: **Single-molecule FRET with diffusion and conformational dynamics**. *J Phys Chem B* 2007, **111**:12925-12932.

27. Gopich IV, Szabo A: **Theory of photon counting in single-molecule spectroscopy**. In *Theory and Evaluation of Single-Molecule Signals.* Edited by Barkai E, Brown FLH, Orrit M, Yang H. World Scientific Pub. Co.; 2009:1-64.

28. Gopich IV, Nettels D, Schuler B, Szabo A: **Protein dynamics from single-molecule fluorescence intensity correlation functions**. *J Chem Phys* 2009, **131**:095102.

29. • Gopich IV, Szabo A: **Decoding the pattern of photon colors in single-molecule FRET**. *J Phys Chem B* 2009, **113**:10965-10973.
A maximum likelihood approach is introduced that allows a direct determination of the time constants for conformational dynamics from the colors and arrival times of recorded photons. This elegant method has been successfully used for the determination of the average transition path time for protein folding [58••].

30. Gopich IV, Szabo A: **FRET efficiency distributions of multistate single molecules**. *J Phys Chem B* 2010, **114**:15221-15226.

31. • Kalinin S, Valeri A, Antonik M, Felekyan S, Seidel CA: **Detection of structural dynamics by FRET: a photon distribution and fluorescence lifetime analysis of systems with multiple states**. *J Phys Chem B* 2010, **114**:7983-7995.
The analysis of photon statistics previously developed by the authors is extended to characterize conformational dynamics on the time scales of

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

diffusion through the confocal volume. A global analysis of transfer efficiency distributions and fluorescence lifetimes allows static and dynamic heterogeneity to be discriminated.

32. Hyeon C, Lee J, Yoon J, Hohng S, Thirumalai D: **Hidden complexity in the isomerization dynamics of holliday junctions**. *Nature Chem* 2012, **4**:907-914.

33. Gopich IV, Szabo A: **Theory of the energy transfer efficiency and fluorescence lifetime distribution in single-molecule FRET**. *Proc Natl Acad Sci USA* 2012, **109**:7747-7752.

34. Laurence TA, Kong XX, Jager M, Weiss S: **Probing structural heterogeneities and fluctuations of nucleic acids and denatured proteins**. *Proc Natl Acad Sci USA* 2005, **102**:17348-17353.

35. Hoffmann A, Kane A, Nettels D, Hertzog DE, Baumgärtel P, Lengefeld J, Reichardt G, Horsley DA, Seckler R, Bakajin O *et al.*: **Mapping protein collapse with single-molecule fluorescence and kinetic synchrotron radiation circular dichroism spectroscopy**. *Proc Natl Acad Sci USA* 2007, **104**:105-110.

36. • Chung HS, Gopich IV, McHale K, Cellmer T, Louis JM, Eaton WA: **Extracting rate coefficients from single-molecule photon trajectories and FRET efficiency histograms for a fast-folding protein**. *J Phys Chem A* 2011, **115**:3642-3656.

A simple and widely applicable method for the characterization of protein dynamics on the time scale of translational diffusion (1 ms) is used to determine the folding times of the designed protein α_3D. The method uses Gaussian distributions to describe the bin-size dependent changes in the FRET efficiency histograms. Since the photon trajectory is not needed [29•], the method is especially useful for simple ratiometric single-molecule FRET experiments.

37. • Hoffmann A, Nettels D, Clark J, Borgia A, Radford SE, Clarke J, Schuler B: **Quantifying heterogeneity and conformational dynamics from single molecule FRET of diffusing molecules: recurrence analysis of single particles (RASP)**. *Phys Chem Chem Phys* 2011, **13**:1857-1871.

A widely applicable method for determining the interconversion kinetics between subpopulations in FRET-efficiency histograms is proposed based on recurrence analysis. Accordingly, at the picomolar sample concentrations used in experiments with freely diffusing molecules and for time scales up to several tens of milliseconds, the probability for a molecule to return to the confocal volume is higher than the probability of detecting a new molecule. Recurrence analysis quantifies this effect and thus significantly extends the dynamic range of the conventional transfer efficiency histogram analysis. Kinetics on time scales from $\sim$50 μs to $\sim$100 ms can be analyzed at equilibrium.

38. Deniz AA, Laurence TA, Beligere GS, Dahan M, Martin AB, Chemla DS, Dawson PE, Schultz PG, Weiss S: **Single-molecule protein folding: diffusion fluorescence resonance energy transfer studies of the denaturation of chymotrypsin inhibitor 2**. *Proc Natl Acad Sci USA* 2000, **97**:5179-5184.

39. Schuler B, Lipman EA, Eaton WA: **Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy**. *Nature* 2002, **419**:743-747.

40. O'Brien EP, Ziv G, Haran G, Brooks BR, Thirumalai D: **Effects of denaturants and osmolytes on proteins are accurately predicted by the molecular transfer model**. *Proc Natl Acad Sci USA* 2008, **105**:13403-13408.

41. Hofmann H, Soranno A, Borgia A, Gast K, Nettels D, Schuler B: **Polymer scaling laws of unfolded and intrinsically disordered proteins quantified with single molecule spectroscopy**. *Proc Natl Acad Sci USA*, **109**, 16155–16160.

42. Nettels D, Müller-Späth S, Küster F, Hofmann H, Haenni D, Rüegger S, Reymond L, Hoffmann A, Kubelka J, Heinz B *et al.*: **Single molecule spectroscopy of the temperature-induced collapse of unfolded proteins**. *Proc Natl Acad Sci USA* 2009, **106**:20740-20745.

43. Best RB, Mittal J: **Protein simulations with an optimized water model: cooperative helix formation and temperature-induced unfolded state collapse**. *J Phys Chem B* 2010, **114**:14916-14923.

44. Müller-Späth S, Soranno A, Hirschfeld V, Hofmann H, Rüegger S, Reymond L, Nettels D, Schuler B: **Charge interactions can dominate the dimensions of intrinsically disordered proteins**. *Proc Natl Acad Sci USA* 2010, **107**:14609-14614.

45. Hofmann H, Golbik RP, Ott M, Hübner CG, Ulbrich-Hofmann R: **Coulomb forces control the density of the collapsed unfolded state of barstar**. *J Mol Biol* 2008, **376**:597-605.

46. Mao AH, Crick SL, Vitalis A, Chicoine CL, Pappu RV: **Net charge per residue modulates conformational ensembles of intrinsically disordered proteins**. *Proc Natl Acad Sci USA* 2010, **107**:8183-8188.

47. Huang F, Rajagopalan S, Settanni G, Marsh RJ, Armoogum DA, Nicolaou N, Bain AJ, Lerner E, Haas E, Ying L *et al.*: **Multiple conformations of full-length p53 detected with single-molecule fluorescence resonance energy transfer**. *Proc Natl Acad Sci USA* 2009, **106**:20758-20763.

48. Lum JK, Neuweiler H, Fersht AR: **Long-range modulation of chain motions within the intrinsically disordered transactivation domain of tumor suppressor p53**. *J Am Chem Soc* 2012, **134**:1617-1622.

49. Milles S, Lemke EA: **Single molecule study of the intrinsically disordered FG-repeat nucleoporin 153**. *Biophys J* 2011, **101**:1710-1719.

50. Sherman E, Haran G: **Coil-globule transition in the denatured state of a small protein**. *Proc Natl Acad Sci USA* 2006, **103**:11539-11543.

51. Ziv G, Thirumalai D, Haran G: **Collapse transition in proteins**. *Phys Chem Chem Phys* 2009, **11**:83-93.

52. Nettels D, Gopich IV, Hoffmann A, Schuler B: **Ultrafast dynamics of protein collapse from single-molecule photon statistics**. *Proc Natl Acad Sci USA* 2007, **104**:2655-2660.

53. Nettels D, Hoffmann A, Schuler B: **Unfolded protein and peptide dynamics investigated with single-molecule FRET and correlation spectroscopy from picoseconds to seconds**. *J Phys Chem B* 2008, **112**:6137-6146.

54. Wahl M, Rahn H-J, Röhlicke T, Kell G, Nettels D, Hillger F, Schuler B, Erdmann R: **Scalable time-correlated photon counting system with multiple independent input channels**. *Rev Sci Instrum* 2008, **79**:123113.

55. Doi M, Edwards SF: *The Theory of Polymer Dynamics*. New York, USA: Oxford University Press; 1988.

56. Khatri BS, McLeish TCB: **Rouse model with internal friction: a coarse grained framework for single biopolymer dynamics**. *Macromolecules* 2007, **40**:6770-6777.

57. Shoemaker BA, Portman JJ, Wolynes PG: **Speeding molecular recognition by using the folding funnel: the fly-casting mechanism**. *Proc Natl Acad Sci USA* 2000, **97**:8868-8873.

58. •• Chung HS, McHale K, Louis JM, Eaton WA: **Single-molecule fluorescence experiments determine protein folding transition path times**. *Science* 2012, **335**:981-984.

The first direct determination of the transition path time for a protein folding reaction. A particularly remarkable result of this *tour-de-force* in single-molecule spectroscopy is that the microsecond transition path times of the two proteins investigated (WW-domain and GB1) differ by less than a factor of five even though their folding times differ by four orders of magnitude. The actual process of folding, that is, the barrier crossing event, is thus rather insensitive to the barrier height.

59. Cellmer T, Henry ER, Kubelka J, Hofrichter J, Eaton WA: **Relaxation rate for an ultrafast folding protein is independent of chemical denaturant concentration**. *J Am Chem Soc* 2007, **129**:14564-14565.

60. Doose S, Neuweiler H, Sauer M: **Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules**. *Chemphyschem* 2009, **10**:1389-1398.

61. Lapidus LJ, Eaton WA, Hofrichter J: **Measuring the rate of intramolecular contact formation in polypeptides**. *Proc Natl Acad Sci USA* 2000, **97**:7220-7225.

62. Noe F, Doose S, Daidone I, Lollmann M, Sauer M, Chodera JD, Smith JC: **Dynamical fingerprints for probing individual relaxation processes in biomolecular dynamics with simulations and kinetic experiments**. *Proc Natl Acad Sci USA* 2011, **108**:4822-4827.

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

63. Neuweiler H, Lollmann M, Doose S, Sauer M: **Dynamics of unfolded polypeptide chains in crowded environment studied by fluorescence correlation spectroscopy**. *J Mol Biol* 2007, **365**:856-869.

64. Neuweiler H, Banachewicz W, Fersht AR: **Kinetics of chain motions within a protein-folding intermediate**. *Proc Natl Acad Sci USA* 2010, **107**:22106-22110.

65. • Neuweiler H, Johnson CM, Fersht AR: **Direct observation of ultrafast folding and denatured state dynamics in single protein molecules**. *Proc Natl Acad Sci USA* 2009, **106**:18569-18574.
The authors investigated a tryptophan-containing variant of the small protein BBL with ns-PET-FCS. In the correlation functions in the absence of denaturant, they observed two submillisecond contributions, one with a decay time of ~10 μs and the other with ~0.5 μs to ~1 μs, which they assigned to folding kinetics and loop closure dynamics in the denatured state, respectively. The solvent viscosity dependence of the loop closure rates provided evidence for internal friction in the denatured state. This work demonstrates the potential of ns-PET-FCS for investigating dynamics occurring at different timescales.

66. Chung HS, Cellmer T, Louis JM, Eaton WA: **Measuring ultrafast protein folding rates from photon-by-photon analysis of single molecule fluorescence trajectories**. *Chem Phys* 2012 http://dx.doi.org/10.1016/j.chemphys.2012.08.005.

67. Rhoades E, Cohen M, Schuler B, Haran G: **Two-state folding observed in individual protein molecules**. *J Am Chem Soc* 2004, **126**:14686-14687.

68. Chung HS, Louis JM, Eaton WA: **Experimental determination of upper bound for transition path times in protein folding from single-molecule photon-by-photon trajectories**. *Proc Natl Acad Sci USA* 2009, **106**:11837-11844.

69. Liu F, Nakaema M, Gruebele M: **The transition state transit time of WW domain folding is controlled by energy landscape roughness**. *J Chem Phys* 2009, **131**:195101.

70. Wensley BG, Batey S, Bone FAC, Chan ZM, Tumelty NR, Steward A, Kwa LG, Borgia A, Clarke J: **Experimental evidence for a frustrated energy landscape in a three-helix-bundle protein family**. *Nature* 2010, **463** 685–U122.

71. Sherman E, Haran G: **Fluorescence correlation spectroscopy of fast chain dynamics within denatured protein L**. *Chemphyschem* 2011, **12**:696-703.

72. Rhoades E, Gussakovsky E, Haran G: **Watching proteins fold one molecule at a time**. *Proc Natl Acad Sci USA* 2003, **100**:3197-3202.

73. • Pirchi M, Ziv G, Riven I, Cohen SS, Zohar N, Barak Y, Haran G: **Single-molecule fluorescence spectroscopy maps the folding landscape of a large protein**. *Nat Commun* 2011, **2**:493.
The authors investigated the folding of the multi-domain protein adenylate kinase by analyzing thousands of single-molecule trajectories with Markov-state models. The results suggest a complex energy landscape for adenylate kinase folding, with about six meta-stable states whose connectivity is strongly denaturant-dependent.

74. Kuzmenkina EV, Heyes CD, Nienhaus GU: **Single-molecule Forster resonance energy transfer study of protein dynamics under denaturing conditions**. *Proc Natl Acad Sci USA* 2005, **102**:15471-15476.

75. Garcia-Mira MM, Sadqi M, Fischer N, Sanchez-Ruiz JM, Muñoz V: **Experimental identification of downhill protein folding**. *Science* 2002, **298**:2191-2195.

76. Huang F, Ying L, Fersht AR: **Direct observation of barrier-limited folding of BBL by single-molecule fluorescence resonance energy transfer**. *Proc Natl Acad Sci USA* 2009, **106**:16239-16244.

77. Liu J, Campos LA, Cerminara M, Wang X, Ramanathan R, English DS, Munoz V: **Exploring one-state downhill protein folding in single molecules**. *Proc Natl Acad Sci USA* 2012, **109**:179-184.

78. Lipman EA, Schuler B, Bakajin O, Eaton WA: **Single-molecule measurement of protein folding kinetics**. *Science* 2003, **301**:1233-1235.

79. Hamadani KM, Weiss S: **Nonequilibrium single molecule protein folding in a coaxial mixer**. *Biophys J* 2008, **95**:352-365.

80. Pfeil SH, Wickersham CE, Hoffmann A, Lipman EA: **A microfluidic mixing system for single-molecule measurements**. *Rev Sci Instrum* 2009, **80**:055105.

81. • Gambin Y, Vandelinder V, Ferreon AC, Lemke EA, Groisman A, Deniz AA: **Visualizing a one-way protein encounter complex by ultrafast single-molecule mixing**. *Nat Methods* 2011, **8**:239-241.
An elegant microfluidic mixing device for non-equilibrium single-molecule kinetics was designed with a dead time of 200 μs. The authors used this device to investigate the SDS-induced folding of α-synuclein, an intrinsically disordered protein, and observed the rapid population of transient structures in the formation of the α-synuclein-SDS complex.

82. Orte A, Craggs TD, White SS, Jackson SE, Klenerman D: **Evidence of an intermediate and parallel pathways in protein unfolding from single-molecule fluorescence**. *J Am Chem Soc* 2008, **130**:7898-7907.

83. Knight JB, Vishwanath A, Brody JP, Austin RH: **Hydrodynamic focusing on a silicon chip: mixing nanoliters in microseconds**. *Phys Rev Lett* 1998, **80**:3863-3866.

84. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM: **Rapid prototyping of microfluidic systems in poly(dimethylsiloxane)**. *Anal Chem* 1998, **70**:4974-4984.

85. Gambin Y, Simonnet C, VanDelinder V, Deniz A, Groisman A: **Ultrafast microfluidic mixer with three-dimensional flow focusing for studies of biochemical kinetics**. *Lab Chip* 2010, **10**:598-609.

86. • Hofmann H, Hillger F, Pfeil SH, Hoffmann A, Streich D, Haenni D, Nettels D, Lipman EA, Schuler B: **Single-molecule spectroscopy of protein folding in a chaperonin cage**. *Proc Natl Acad Sci USA* 2010, **107**:11793-11798.
The advantage of single-molecule spectroscopy to work under conditions of nearly infinite dilution was used together with microfluidics to investigate the folding of a highly aggregation-prone enzyme, rhodanese, in free solution and inside the confining cavity formed by the chaperonin GroEL/ES from milliseconds to hours. The surprising finding of a deceleration of folding inside the confined space of the GroEL/ES cavity challenges the concept that confinement universally accelerates folding *in vivo*.

87. Sharma S, Chakraborty K, Muller BK, Astola N, Tang YC, Lamb DC, Hayer-Hartl M, Hartl FU: **Monitoring protein conformation along the pathway of chaperonin-assisted folding**. *Cell* 2008, **133**:142-153.

88. Shtilerman M, Lorimer GH, Englander SW: **Chaperonin function: folding by forced unfolding**. *Science* 1999, **284**:822-825.

89. Jia YW, Talaga DS, Lau WL, Lu HSM, DeGrado WF, Hochstrasser RM: **Folding dynamics of single GCN4 peptides by fluorescence resonant energy transfer confocal microscopy**. *Chem Phys* 1999, **247**:69-83.

90. Rasnik I, McKinney SA, Ha T: **Nonblinking and long-lasting single-molecule fluorescence imaging**. *Nat Methods* 2006, **3**:891-893.

91. Campos LA, Liu J, Wang X, Ramanathan R, English DS, Munoz V: **A photoprotection strategy for microsecond-resolution single-molecule fluorescence spectroscopy**. *Nat Methods* 2011, **8**:143-146.

92. Ha T, Tinnefeld P: **Photophysics of fluorescent probes for single-molecule biophysics and super-resolution imaging**. *Annu Rev Phys Chem* 2012, **63**:595-617.

93. Chung HS, Louis JM, Eaton WA: **Distinguishing between protein dynamics and dye photophysics in single-molecule FRET experiments**. *Biophys J* 2010, **98**:696-706.

94. • Choi UB, McCann JJ, Weninger KR, Bowen ME: **Beyond the random coil: stochastic conformational switching in intrinsically disordered proteins**. *Structure* 2011, **19**:566-576.
Using camera-based TIRF, the authors investigated five different IDPs with different sequence composition and found that two of them exhibit conformational dynamics with time constants in the seconds range. Even though the underlying mechanism is unclear, this is the first reported case of slow dynamics in IDPs in the absence stabilizing ligands (compared to the submicrosecond dynamics of unfolded proteins [25•,52]).

95. Betancourt MR, Thirumalai D: **Exploring the kinetic requirements for enhancement of protein folding rates in the GroEL cavity**. *J Mol Biol* 1999, **287**:627-644.

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008

96. Jewett AI, Baumketner A, Shea JE: **Accelerated folding in the weak hydrophobic environment of a chaperonin cavity: creation of an alternate fast folding pathway**. *Proc Natl Acad Sci USA* 2004, **101**:13192-13197.

97. Takagi F, Koga N, Takada S: **How protein thermodynamics and folding mechanisms are altered by the chaperonin cage: molecular simulations**. *Proc Natl Acad Sci USA* 2003, **100**:11367-11372.

98. • Borgia MB, Borgia A, Best RB, Steward A, Nettels D, Wunderlich B, Schuler B, Clarke J: **Single-molecule fluorescence reveals sequence-specific misfolding in multidomain proteins**. *Nature* 2011, **474**:662-665.
In a combination of protein engineering and single-molecule spectroscopy, the authors provide evidence for an evolutionary mechanism to prevent misfolding in multi-domain proteins. Tandem arrays of immunoglobulin-like domains only showed a misfolded population if adjacent domains had a high sequence identity. The misfolded species was shown to convert to folded proteins with a half-life of 100 hours, demonstrating that single-molecule FRET can be used to investigate very slow conformational changes.

99. Oberhauser AF, Marszalek PE, Carrion-Vazquez M, Fernandez JM: **Single protein misfolding events captured by atomic force microscopy**. *Nat Struct Biol* 1999, **6**:1025-1028.

100. Wright CF, Teichmann SA, Clarke J, Dobson CM: **The importance of sequence diversity in the aggregation and evolution of proteins**. *Nature* 2005, **438**:878-881.

101. Gambin Y, Deniz AA: **Multicolor single-molecule FRET to explore protein folding and binding**. *Mol Biosyst* 2010, **6**:1540-1547.

102. Schuetz P, Wuttke R, Schuler B, Caflisch A: **Free energy surfaces from single-distance information**. *J Phys Chem B* 2010, **114**:15227-15235.

103. Kamagata K, Kawaguchi T, Iwahashi Y, Baba A, Fujimoto K, Komatsuzaki T, Sambongi Y, Goto Y, Takahashi S: **Long-term observation of fluorescence of free single molecules to explore protein-folding energy landscapes**. *J Am Chem Soc* 2012, **134**:11525-11532.

104. Lemke EA: **Site-specific labeling of proteins for single-molecule FRET measurements using genetically encoded ketone functionalities**. *Methods Mol Biol* 2011, **751**:3-15.

105. Chakraborty A, Wang DY, Ebright YW, Ebright RH: **Azide-specific labeling of biomolecules by Staudinger-Bertozzi ligation: phosphine derivatives of fluorescent probes suitable for single-molecule fluorescence spectroscopy**. *Methods in Enzymology, Vol 472: Single Molecule Tools, Pt A: Fluorescence Based Approaches*. 2010:. 19–30.

106. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B *et al.*: **Real-time DNA sequencing from single polymerase molecules**. *Science* 2009, **323**:133-138.

107. Zappa F, Tisa S, Tosi A, Cova S: **Principles and features of single-photon avalanche diode arrays**. *Sens. Actuators A: Phys.* 2007, **140**:103-112.

108. Gambin Y, Schug A, Lemke EA, Lavinder JJ, Ferreon ACM, Magliery TJ, Onuchic JN, Deniz AA: **Direct single-molecule observation of a protein living in two opposed native structures**. *Proc Natl Acad Sci USA* 2009, **106**:10153-10158.

109 • Sakon JJ, Weninger KR: **Detecting the conformation of individual proteins in live cells**. *Nat Methods* 2010, **7**:203-205.
The authors present the first application of single-molecule FRET to conformational changes of proteins inside living cells. Using objective-type TIRF in combination with single-particle tracking and microinjection, they monitored individual proteins incorporating into folded complexes at the cell membrane. This work provides a first important step towards investigating protein folding with single-molecule spectroscopy *in vivo*.

110. Gelman H, Platkov M, Gruebele M: **Rapid perturbation of free-energy landscapes: from in vitro to in vivo**. *Chemistry* 2012, **18**:6420-6427.

111. Phillip Y, Kiss V, Schreiber G: **Protein-binding dynamics imaged in a living cell**. *Proc Natl Acad Sci USA* 2012, **109**:1461-1466.

Please cite this article in press as: Schuler B, Hofmann H. Single-molecule spectroscopy of protein folding dynamics—expanding scope and timescales, Curr Opin Struct Biol (2013), http://dx.doi.org/10.1016/j.sbi.2012.10.008