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TOPICAL REVIEW

Single-molecule fluorescence spectroscopy of biomolecular folding

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Abstract

Single-molecule fluorescence spectroscopy is emerging as an important tool for studying biomolecular folding dynamics. Its usefulness stems from its ability to directly map heterogeneities in folding pathways and to provide information about the energy landscape of proteins and ribonucleic acid (RNA) molecules. Single-molecule fluorescence techniques relevant for folding studies, including methods for trapping and immobilizing molecules, are described and compared in this review. Some emphasis is placed on fluorescence resonance energy transfer, which is particularly useful for studying conformational dynamics of biomolecules. Studies on protein and RNA folding using this methodology are reviewed and set in the more general context of folding science. Finally, some of the interesting future prospects in this field are delineated.

(Some figures in this article are in colour only in the electronic version)

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1. Introduction

Folding is the process by which biological macromolecules, mainly proteins and ribonucleic acids (RNA), attain the three-dimensional structure required for their function. Biomolecular folding science has undergone an enormous change in the last ten years. This change has been driven both by the introduction of a new theoretical framework and by the development of new experimental techniques. These advances have led to a deeper understanding of the physical determinants of folding. One of the major new insights has to do with an understanding of the role of statistical mechanics in the process. More specifically, it is now believed that, rather than going from an unfolded to the folded conformation via a unique pathway, a macromolecule may sample any of an ensemble of structures at each step of the reaction. Analysing such a complex and heterogeneous sequence of events requires spectroscopic tools that can overcome the averaging inherent in standard techniques. Single-molecule spectroscopy holds the promise of dissecting the complexity of the folding process, and work in recent years has started to show that this can indeed be achieved. Two major techniques have been applied to study folding on the single-molecule level. The first, which has been reviewed recently in this journal [1], is force spectroscopy, in which a macromolecule is mechanically unfolded by pulling on it using laser tweezers, magnetic tweezers or an atomic force microscope. This technique can provide interesting insights about the energy landscape of the unfolding reaction coordinate under mechanical force [2]. Especially fruitful is the combination of force spectroscopy with protein engineering methodology and numerical simulation, leading to a characterization of the mechanical unfolding transition state [3]. The second technique, single-molecule fluorescence spectroscopy, allows the researcher to measure the fluorescent signal from an individual molecule and analyse it to get information about the conformational dynamics involved in folding. This topical review will focus only on this latter technique. Its purpose is to examine the budding field of single-molecule fluorescence spectroscopy of folding, describe the methodology used, put in context the findings to date and point to possible future directions.

The review is organized as follows: sections 1.1 and 1.2 provide a rather brief introduction to the modern view of the folding problem, in the cases of proteins and RNA, respectively. Section 2 discusses the essentials of single-molecule fluorescence spectroscopy. Section 2.1 introduces the experimental techniques used, from spectroscopy on diffusing molecules to spectroscopy on immobilized molecules. Chemical methods for molecular immobilization on surfaces are also discussed. In section 2.2 some of the challenges involved in the analysis of single-molecule data are briefly presented. Section 3 focuses on fluorescence resonance energy transfer (FRET) as the major tool for studying biomolecular folding on the single-molecule level, while section 4 reviews literature findings on protein and RNA folding using FRET. Section 5 concludes with a discussion of some of the issues that might occupy this field in the coming years.

1.1. Protein folding

Proteins are heteropolymers, each consisting of a particular sequence of the 20 possible monomers, the amino acids. Proteins typically form a well-defined three-dimensional structure under equilibrium native conditions, i.e. under the conditions that allow them to function

optimally. It has been known since the seminal experiments of Anfinsen [4] that most proteins can spontaneously fold to their native structure. The process of folding was shown to be an intramolecular first-order phase transition [5]. In some cases only two phases are involved in the process, namely the unfolded and the folded forms of the protein. In chemical terminology such a process is called a 'two-state reaction'. The reaction coordinate in this case involves a single, well-defined transition state, which is the fleeting structure existing at the top of the free energy barrier separating the two states [6, 7]. Formation of the transition state is equivalent to nucleation. There are cases where additional phases are involved and a phase diagram can be constructed which delineates their existence as a function of external conditions such as temperature or the presence of chaotropic agents (chemicals that stabilize partially structured forms of the protein) [8]. These intermediate phases can also be seen sometimes in kinetic experiments of folding. For many years it was believed that each protein possesses a unique folding pathway from the unfolded (sometimes called the denatured) to the folded protein, involving a discrete number of intermediate structures.

In recent years a statistical–mechanical theory of folding has been put forward by several authors, who stressed the involvement of ensembles of structures and of multiple pathways connecting them during the folding process [9–11]. Folding is described within this framework as the motion of the protein on a high-dimensional energy landscape. As more and more of the chain contacts that exist in the folded state (termed 'native contacts') are formed during the folding process, the structure becomes increasingly stabilized. Therefore the energy landscape of the folding protein is sometimes depicted as a funnel, with the native state at its bottom [12, 13]. Since there are fewer conformational states that the protein has access to as it moves down the energy funnel, an entropic bottleneck may occur, leading to kinetic folding barriers. At least two temperatures describe the folding process [9]. The first is the folding temperature, i.e. the temperature under which a protein is thermodynamically predisposed to transfer into the folded state. The second temperature is the glass transition temperature, which is related to the local roughness of the energy landscape of the protein. As the protein passes through the glass transition the energy landscape becomes so rugged that dynamics on this surface are too slow for efficient folding. Therefore a 'good' folder is a protein whose folding temperature is significantly higher than the glass transition temperature. A third important temperature is related to the initial collapse of the heteropolymer chain as the solvent conditions are changed from good to bad [11]. This collapse may either precede or coincide with the folding transition itself. Small proteins are expected to have a smooth energy landscape and therefore fold quickly and without populating intermediate states (or phases) between the folded and unfolded states. Larger proteins are, in general, expected to possess a rough energy landscape and their folding process is dominated by recovery out of traps, or misfolded intermediate states [14].

This theoretical picture of folding has been corroborated by experimental work on many different levels. Many small proteins clearly show only two thermodynamic states, folded or unfolded, and their folding kinetics are dominated by barrier crossing [15]. Fersht and co-workers developed a biochemical method, ϕ-value analysis, which allows structural characterization of the folding transition state (or nucleus). The method is based on comparison of the effect of single-site mutations on the rate of folding and the overall stability of the protein [16]. From a structural point of view studies on small proteins suggest that formation of secondary structure is concomitant with the formation of tertiary structure [17]. In other words, stabilization of secondary structure elements requires the formation of tertiary contacts. There are cases, though, where secondary structure elements are stable enough to form first and then the folding process is more likely to involve a search for the proper three-dimensional organization of these elements. Such a process, which was described by Weaver and Karplus

as the diffusion–collision mechanism [18], may lead to extremely fast folding rates, as has been shown recently [19].

Several techniques have been devised for the fast initiation of folding, which allows a study of the very early events during the process [20]. These methods include ultrarapid mixing [21], temperature jump [22] and optical triggering [23]. Using such techniques has allowed researchers to expose deviations from simple exponential kinetics in the folding of several small proteins and peptides [24–26]. Nonexponential relaxation may point to the involvement of multiple folding pathways, or alternatively reveal those aspects of the motion on the energy landscape that do not strictly involve a crossing of the folding energy barrier. In particular, it is of interest to characterize the diffusional motion of the protein chain and understand the factors determining this motion, mainly having to do with the local roughness of the energy landscape. This diffusional motion enters the rate of folding through the pre-exponential in the transition state theory expression for the rate

$$k_{\text{folding}} = k_0 \exp(-\Delta G^{\#}/k_{\text{B}}T)$$

in which $\Delta G^{\#}$ is the folding free energy barrier [27]. The pre-exponential, k_0, can be modelled using Kramers' theory of barrier crossing [28] and is related to the rate of intrachain diffusion. This rate has been experimentally inferred from ensemble FRET measurements on short peptides [29], and more recently in experiments using triplet quenching to study kinetics of contact formation between two sites on a polypeptide [30, 31]. These experiments suggest that k_0 may be as large as 10^7 s^{-1}, setting an upper limit for the folding rate. It is possible, though, that in larger proteins, where the energy landscape is significantly rougher, the rate of intrachain diffusion may take a much smaller value. Further, this rate might be significantly coordinate-dependent [27]. Such variability cannot be gleaned directly from ensemble experiments, while single-molecule experiments have the potential to probe it, by directly visualizing the motion of an individual protein molecule at different positions on its energy surface.

As has been noted above, theory suggests that the folding pathway and rate should depend on the initial conditions. What are the initial conditions for folding? Experiments in recent years suggest that many proteins do not adopt a fully denatured conformation in their unfolded state [32]. Experiments which probe global structure may indicate that an unfolded protein is a random coil, while experiments using more local probes show significant residual structure even under highly denaturing conditions. A well-studied case is that of staphylococcal nuclease. A large fragment of this protein was shown not to fold under native conditions and could thus serve as a model for the denatured state [33]. A significant amount of long-range structure was found in this fragment by NMR techniques [34], with overall topology quite similar to the native topology, even under strongly denaturing conditions [35]. A compact structure or residual secondary structure elements were found in the unfolded states of other proteins as well [36, 37].

Modulation of the folding pathway by variation of the initial conditions has been directly demonstrated in several cases. For example, protein G shows strictly two-state folding kinetics when folded at neutral pH, yet it populates a folding intermediate when folded at acidic pH and high salt concentration [38]. Even more dramatically, the folding kinetics of another protein, the WW domain, could be tuned between two-state and three-state kinetics by either small temperature changes or mutation [39].

1.2. RNA folding

The RNA folding problem has received serious attention only in recent years [40], following the realization that RNA molecules can possess autocatalytic activity [41]. The vocabulary

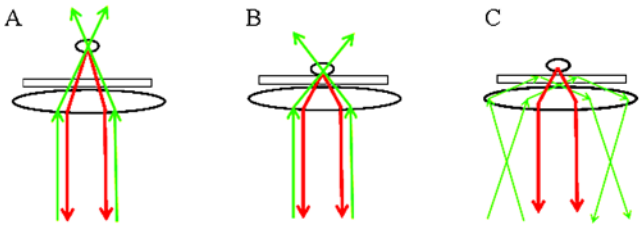

Figure 1. Optical configurations of three common single-molecule fluorescence techniques. (A) Spectroscopy of freely diffusing molecules. The excitation laser beam, in green (grey), is focused within the experimental chamber to define a diffraction-limited volume. Fluorescence, in red (black), is collected only from molecules passing through this volume. (B) Spectroscopy of surface-immobilized molecules. The excitation beam now forms a diffraction-limited spot which is scanned over the surface to identify individual molecules and then collect fluorescence photons from them. (C) Objective-type total internal reflection microscopy (TIRFM). The excitation beam is totally reflected at the interface between glass and water. Molecules adsorbed at this interface are excited by the evanescent wave penetrating into the experimental chamber and their fluorescence is collected with the same objective used for excitation. A second arrangement for TIRFM, where the excitation beam is introduced through a prism, is not shown.

of RNA sequences is more limited than that of proteins, since there are only four monomers, the nucleotides. Conceptually, the main difference between protein and RNA folding lies in the fact that, while the folding of the former is driven mainly by hydrophobic interactions between side-chains, the latter strongly depends on charge neutralization caused by counterion condensation. Indeed, an initial stage of counterion condensation drives a collapse of the RNA chain in a similar fashion to the collapse experienced by a protein chain in the initial stages of folding [42]. Another unique feature of RNA folding is the stable formation of secondary structures even in the absence of tertiary interactions [40], which is generally not true in proteins. This fact suggests that a diffusion–collision-like model [18] might describe major aspects of the RNA folding problem. The folding process of large RNA molecules seems to occur on a rough energy landscape and therefore involves many kinetic traps. Thus, while one part of the Tetrahymena ribozyme (the P4–P6 domain) can form in several seconds, other parts form much more slowly [43]. A kinetic partitioning mechanism, under which part of the population folds in a direct, two-state mechanism while the rest is trapped in misfolded intermediates, can explain the kinetic data on large RNA folding [42]. This situation is probably not very different than that of larger protein molecules. In contrast, small RNA molecules may form their tertiary structure in subsecond times [44].

2. Single-molecule fluorescence spectroscopy—some general methodological considerations

Several related single-molecule spectroscopy techniques were used to study macromolecular folding. The principles of these techniques as well as the pros and cons of each are discussed in section 2.1, including a short description of the various modalities of fluorescence spectroscopy. Molecular immobilization methods relevant to single molecule studies are described in section 2.2. Some aspects of the analysis of single-molecule measurements are presented in section 2.3.

2.1. Experimental techniques

2.1.1. Spectroscopy of freely diffusing molecules (figure 1(A)). In this technique a laser is focused into a dilute solution of the freely diffusing molecule of interest. This is a convenient

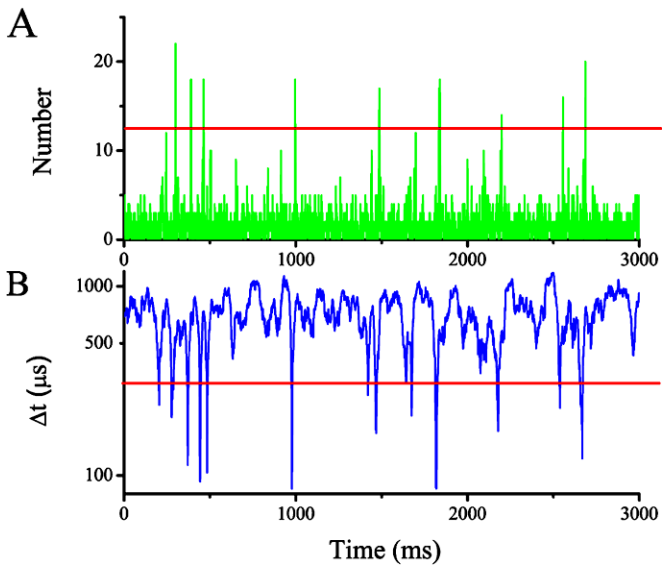

Figure 2. Data from freely diffusing molecules can be collected either by binning on-the-fly using a multichannel scaler (A) or by registering the lag times between photon arrivals at the detector (B). A proper choice of a threshold (marked by a red (black) horizontal line in each plot) identifies a similar set of bursts in the two cases.

technique for obtaining distributions of the properties of single molecules rather quickly, although in the typical experiment dynamic information cannot be directly obtained. A confocal optical set-up is used in order to limit the observation volume in a liquid sample to $<1\ \mu m^3$. This is achieved by inserting a pinhole in the image plane of the microscope, which efficiently rejects out-of-focus light [45]. If the concentration of fluorescing molecules is kept in the picomolar range then the probability of having one molecule in the focal volume of the laser beam is $\sim$0.01. Thus molecules rarely pass through the focus and, when a molecule does traverse the beam a burst of photons is emitted. A single-photon counter detects this burst of photons. Detection is typically done with single-photon-counting avalanche photodiodes, as these devices show a high quantum yield as well as a fast temporal response. The number of photons detected during a single burst, i.e. a single pass of a molecule through the laser beam, depends on several factors. First and foremost is the dwell time, which is the overall time it takes a molecule to cross the illuminated region. The dwell time is distributed exponentially [46], and for a typical confocal set-up its average value is of the order of milliseconds. The laser power used and the photophysics of the molecule will also affect the number of photons collected, but typically several tens of photons are detected. A thorough analysis of the statistics of fluorescence bursts can be found in [47] and references therein.

There are two possible ways to collect photons in this experiment. First, the photons can be binned on the fly into time bins with predefined widths, using a multichannel scaler or similar device. In this case a photon burst is discriminated from the background by setting a certain threshold, which is defined according to the statistics of the measurement (figure 2(A)). A second option is to measure the relative arrival time of photons at the detector [48]. A trajectory of arrival times is then obtained and the passage of a molecule through the beam is marked by a sharp reduction in the time lag between photons (figure 2(B)). The advantage of this method is that it makes use of the full time resolution of the detectors, a factor which might

be important, for example, if one would like to calculate correlation functions from the data. In fact, it is straightforward to convert an arrival time trajectory into a multichannel scaler-like trajectory.

Several modifications are possible that make the type of experiment described above even more informative. First, a pulsed source of excitation can be used in place of a CW source, allowing for the time delay between a pulse and the arrival of an emitted photon at the detector to be measured. This type of experiment is called time-correlated single-photon counting. By constructing histograms of photon delay times one can obtain the fluorescence lifetime. Seidel and co-workers [49] showed that this method can be combined with measurements on diffusing molecules and that the fluorescence lifetime of a single molecule can be obtained from as little as 100 photons.

Another modification of the free diffusion experiment is achieved by flowing molecules in capillaries [50], or in the channels of microfluidic devices [51]. Flowing can improve the sampling rate of single molecules in solution. Further, devices that quickly mix two solutions can be constructed [52], thereby opening the way to non-equilibrium experiments. Such devices have been used in recent years in ultrarapid (submillisecond) ensemble folding experiments [53], and efforts to apply them to single-molecule studies are under way [54]. Finally, the dwell time of diffusing molecules in the beam can be increased, either by using viscogenic agents to slow down the diffusion, or by actually immobilizing the molecules on a surface. The spectroscopy of immobilized molecules constitutes a major branch of the methodology of single-molecule fluorescence and the next sections are dedicated to it.

2.1.2. Spectroscopy of immobilized molecules. Two basic methods are used for studying individual immobilized molecules. The first technique is a variant of confocal microscopy in which the molecules are not diffusing in solution but are attached on a glass surface (figure 1(B)). Typically, the surface is scanned in order to identify fluorescent molecules. Photons are collected from each molecule until it photobleaches. As above, avalanche photodiodes are used as detectors. Typically, a single molecule emits $\sim$1 million photons before photobleaching, out of which up to 10% can be detected. Again, the detected photons can be binned on-the-fly or else their arrival times at the detector can be recorded [55]. Yang and Xie [56, 57] have recently elaborated the methodology for analysis of photon arrival trajectories and showed how correlation functions can be used to significantly improve the time resolution of single-molecule experiments.

The second technique is total internal reflection fluorescence microscopy (TIRFM) [58]. A laser beam is totally reflected at the interface between glass and the water comprising the sample, and an evanescent wave is formed within the sample. The excitation light is introduced either through a prism [59] or through the objective [60] (figure 1(C)). Specialized objectives with numerical apertures of 1.45 and 1.65 can be used for the latter configuration. The evanescent wave in TIRFM excites molecules that are either at the surface or within $\sim$100 nm of the surface. There are two advantages to this type of illumination. First, if the interaction of a diffusing fluorescent molecule with a molecule adsorbed on the surface is studied, then the fluorescent molecule concentration can be kept rather high without introducing a large background signal [61]. This advantage is not so important for the typical folding experiment. A second benefit of TIRFM is the ability to illuminate a large area on the surface, even in excess of 50 μm in diameter. Thus many adsorbed molecules are illuminated at once. The signals from these molecules can be collected using a CCD camera, and so a large number of trajectories can be obtained in a short time. It should be noted though that the time resolution of a CCD camera is significantly lower than that of an avalanche photodiode, which limits the experiment to slower events.

Very recently, Webb and co-workers [62] introduced a new technique akin to TIRFM. In this technique a thin layer of metal is deposited on the glass surface and holes are made in it. Light impinging on this structure cannot penetrate through the holes since they do not support propagating modes of the electromagnetic field. An evanescent field is formed in each of the holes, leading to excitation of a very small volume, smaller by a factor of $\sim$1000 than the resolution volume in TIRFM.

2.1.3. Modalities of fluorescence spectroscopy. While several different types of fluorescence spectroscopy have been used for the study of individual biomolecules, folding studies have almost exclusively relied on the use of FRET, with the exception of one study, in which fluorescence self-quenching was used as a probe of folding of a protein heavily labelled with fluorophores [63]. A thorough discussion of FRET will be deferred to section 3. For the sake of completeness I will briefly discuss here other common fluorescence techniques as they are applied to single-molecule spectroscopy.

The simplest observable in fluorescence spectroscopy is the emission intensity. The time dependence of the intensity can yield important information about the biomolecular dynamics. As an example note the work of Xie and co-workers [64] in which an enzymatic cycle was probed by following changes in fluorescence intensity of a fluorescent flavin cofactor. Another example is the blinking of fluorescent proteins, which is a manifestation of the complex photophysics of their intrinsic chromophore [65, 66]. Intensity fluctuations mirror changes in the quantum yield of a fluorophore, which can sometimes be obtained more accurately from measurements of fluorescence lifetimes [49]. Another manifestation of the time-varying interaction of a fluorophore with its environment is spectral fluctuations [67].

The reorientational motion of a fluorophore is one of the richest sources of dynamic information, and it can be followed using fluorescence polarization techniques [68, 69]. By modulating the polarization of the excitation light, splitting the emitted light into two polarization components or a combination of the two, one can obtain accurate information about the orientation of an individual fluorophore. A particularly illuminating example is provided by the recent study of Goldman and co-workers [70], in which a special bidentate fluorophore was rigidly attached to the protein myosin V and its three-dimensional orientation was followed in time to show switching between two conformations during translational jumps on actin. Polarization spectroscopy can also be performed on diffusing molecules, using either CW [71] or pulsed excitation [72], and information about the rotational freedom of a fluorophore attached to a biomolecule can be obtained. This information might be important for the evaluation of FRET data (see section 3).

2.2. Immobilization methods

The spectroscopic methods described in the previous section, (excluding measurements on freely diffusing molecules) require immobilization of the molecules on a surface, sometimes at a specific orientation. Many techniques have been devised in order to bind macromolecules on surfaces, single-molecule experiments being only one of many incentives for their development. Here we will discuss some of those techniques that have been employed to study biomolecular dynamics.

Biomolecules tend to adsorb passively to surfaces typically used in the lab, such as glass (figure 3(A)). This adsorption is based on short-range van der Waals forces or longer-range electrostatic interactions between a molecule and the surface [73]. While passive adsorption provides the simplest means to prepare a sample for single-molecule spectroscopy, it does not allow for control of the orientation of an adsorbed molecule. Furthermore, in some cases the

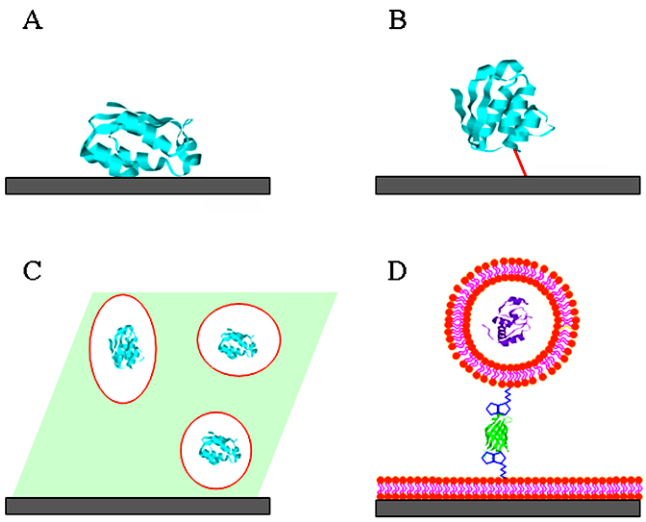

Figure 3. Some methods for immobilization of biomolecules at the water–glass interface. (A) Passive adsorption, relying on non-specific weak interactions between the molecule and the surface. (B) Specific tethering using covalent bonding or non-covalent linking, e.g. with avidin and biotin or with nickel and a histidine tag. This method allows for a specific orientation of the adsorbed molecule to be maintained. (C) Trapping in the pores of a hydrated gel, such as agarose or silica. (D) Trapping within the water compartment of a surface-tethered lipid vesicle, which allows minimal interference with the conformational equilibrium and dynamics of a molecule.

interaction of a macromolecule with the surface can lead to partial denaturation, for example through stabilization of intermediate structures that expose hydrophobic surfaces.

In cases where the orientation of the molecule on the surface is important, it is possible to use specific tethering methods in order to control the way a biomolecule is attached to the surface. A simple, but elegant, method is based on the introduction of a sequence of charged residues into the molecule, and makes use of the specific electrostatic interactions between that segment and a treated surface to bind the molecule in a defined orientation. Jia *et al* [74] used such a technique to attach a polypeptide to a glass surface. The peptide was constructed with a patch of negatively charged amino acids built into it, allowing for its specific adsorption to a positively charged amino-silane treated surface. It is also possible to covalently attach a macromolecule to a surface using a linker with a functional group that is then reacted with the macromolecule (figure 3(B)). A common technique for the introduction of linkers onto a surface is the preparation of a self-assembled monolayer or a similar structure on the surface [75]. Bonding of the functional group to a macromolecule is then achieved by flowing in the macromolecule in the presence of a chemical coupler. For example, a self-assembled monolayer with carboxy groups on the surface can be used to immobilize proteins by forming a peptide bond between these groups and amino side chains of the protein [76]. This and similar techniques are used extensively in the context of surface plasmon resonance spectroscopy [77] and quartz crystal microbalance sensing [78] of biomolecular interactions.

Biotin–avidin chemistry has also been employed to attach biomolecules specifically to surfaces [79]. This method relies on the extremely strong interaction between biotin and the proteins avidin or steptavidin. A biotin moiety is introduced to the surface of the macromolecule that needs to be immobilized. A ‘sandwich’ structure is formed by preparing a biotinilated surface, covering it with avidin molecules and finally attaching biotinilated macromolecules to the avidin.

Another useful approach is to employ the strong interaction between nickel ions and multi-histidine sequences in proteins. This interaction is commonly used in protein purification protocols; a tail including 6–10 histidine residues is introduced into the amino-terminus of a protein and the protein is then purified on an affinity column containing nickel ions. The same method can be used to immobilize protein molecules on nickelated surfaces. Single-molecule studies of rotational motion of the protein F_1-ATPase have utilized this technique [80].

A completely different approach involves the trapping of biomolecules within the pores of gels or glasses (figure 3(C)). A thin layer of a gel, with fluorescent molecules already incorporated, can be prepared on a surface [64, 81–83]. An advantage of gels is that they usually contain a large fraction of water, thus providing a suitably hydrating environment for molecules trapped within them. Further, the size of the pores in these gels can, in principle, be engineered to match the size of the trapped proteins. The compatibility of gels with biomolecules was demonstrated nicely in a recent study on green fluorescent protein molecules trapped in a silica gel, which were found to rotate in a similar fashion to their rotation in free solution, even as their translational motion was essentially frozen [82].

While the above methods can provide a suitable means for immobilizing biomolecules for functional studies on the single-molecule level, it is difficult to apply these methods in the case of folding studies. The main difficulty arises from surface interactions of partially unfolded states of a macromolecule. Such interactions can significantly modify the energy landscape of a protein, as shown by Hochstrasser and co-workers [84]. In fact, even the pure entropic effect of confinement in a cavity on the thermodynamics and kinetics of folding can be significant [85, 86]. Interestingly, RNA molecules seem to suffer less from this problem and their adsorption on a surface does not change their properties significantly [87].

An immobilization method which might overcome this difficulty was recently developed [71]. The method is based on the trapping of single protein molecules within 100 nm lipid vesicles prepared by extrusion and tethered to glass using biotin–avidin chemistry (figure 3(D)). The large size of the vesicles compared to a protein allows ample volume for free rotational motion. This has been shown using a single-molecule fluorescence polarization technique [71]. Further, single-molecule measurements of the FRET efficiency distributions (see section 3) of labelled molecules of the protein adenylate kinase molecules under native and fully denatured conditions showed that these distributions are consistent with ensemble measurements [88], and so the influence of the vesicles on the energy landscape of the protein is probably minimal. Single-molecule folding studies of vesicle-trapped proteins will be discussed in section 4.

2.3. Analysis of single-molecule trajectories

The analysis of noisy single-molecule trajectories presents challenges that researchers are only starting to tackle. It should be noted though that some of the pertinent issues were already dealt with in the literature of single ion-channel recording [89], a technique which has been around for more than 20 years. Here we will focus briefly on two aspects of the problem: the reduction of noise in time-dependent trajectories and the extraction of rate constants from stochastic trajectories. It is possible to devise several different types of filters that will reduce the noise in trajectories and expose some of the information that might be hidden behind this noise, while causing minimal distortion to the data. The simplest procedure that can be applied is a running-average procedure, in which each data point in the averaged trajectory is represented by the average of N points preceding it in the original trajectory. This particular realization of the running average can be termed a 'forward-averaged predictor', and similarly a backward-averaged predictor can be constructed by representing each data point by the average

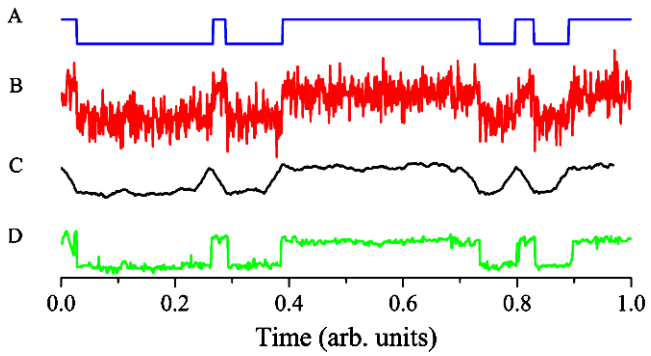

Figure 4. Filtration of a single-molecule trajectory with the non-linear forward–backward filter. (A) Simulated data showing stochastic transitions between two states. (B) The same data with added noise. (C) A running-average filter can reduce the noise significantly but it also distorts the rising and falling edges of the transitions. (D) The adaptive non-linear filter recovers the original data without distorting sharp transitions.

of N points following it. The running-average procedure is one example of a low-pass filter. A more sophisticated linear procedure, the so-called Wiener filter, was used by Talaga *et al* [84]. This filter is applied in Fourier space and uses knowledge about the power spectrum of the photon noise to optimally filter it out [90].

Unfortunately, all linear low-pass filters tend to smear and even distort fast jumps in the data. A forward–backward non-linear filter developed by Chung and Kennedy [91] was recently used by Rhoades *et al* [88] to analyse single-molecule trajectories. This filter builds on the simple running-average procedure and significantly improves it by generating a set of forward-averaged and backward-averaged predictors and adaptively weighting them in a way that ensures that fast jumps in the data are not smeared. The adaptive weights are derived in [91] using Bayesian statistics. An example of the result of operation of this filter on a simulated data set is given in figure 4. It is seen that the filter reconstructs the data out of the noise in a way that allows clear identification of transitions. Other methods to expose data under noise that are extensively used in the single-channel recording community, such as hidden Markov techniques [92], are still waiting to be tested on single-molecule fluorescence data.

A detailed analysis of conformational fluctuations in single-molecule folding trajectories requires a model to describe the underlying dynamics. One way to describe single-molecule dynamics relies on partitioning of the molecular conformational space into discrete states, with rate constants that describe the transitions between them. The problem then reduces to the evaluation of these rate constants from stochastic trajectories. If the various states can be clearly delineated in each trajectory, which is the situation when transitions between states are slow compared to the time resolution of the experiment, then the evaluation of the rate constants can be done using histograms of dwell times in the various states (see, e.g., [64]) or by computing certain statistical moments from the trajectories [93–95]. More generally, rate constants can be evaluated by computing correlation functions of the experimental signals [56, 96], a method akin to the established fluorescence correlation spectroscopy [97].

When the conformational space is not easily divided into separate regions with high barriers between them, a different category of models may be applicable, in which the dynamics is described in terms of a continuous stochastic coordinate, such as a Brownian oscillator [56, 98–100]. Wolynes, Wang and co-workers [101, 102] developed a statistical approach particularly for the analysis of single-molecule folding experiments, trying to answer the question of

which of the two categories of models is appropriate for folding, i.e. whether this process should be described by a set of discrete states or by a continuous coordinate. It was suggested that deviation of folding trajectories from Poisson statistics, as particularly expected close to the glass transition temperature, can be revealed by analysis of higher-order moments of distributions and high-order correlation functions [102, 103].

3. Fluorescence resonance energy transfer (FRET) in single-molecule spectroscopy

We will discuss here in some detail the essentials of FRET and how it is applied to single-molecule spectroscopy (a more general review of the modern application of FRET in biophysics can be found in [104]). The usefulness of this method stems from the fact that it provides a direct access to a real-space distance within a molecule. FRET spectroscopy thus projects all the dynamics of a biomolecule onto a single distance coordinate. FRET was discovered in 1948 by Förster [105], who showed that the transition dipole–transition dipole interaction between two molecules, a donor and an acceptor, can lead to an efficient transfer of excitation energy from one molecule to the other. Using Fermi's golden rule he showed that the rate of energy transfer could be written in the following form:

$$k_{ET} = \frac{1}{\tau}\left(\frac{R_0}{R}\right)^6$$

where τ is the fluorescence lifetime of the donor molecule, R is the distance between the two molecules and R_0, the 'Förster distance', is a constant that depends on their spectral characteristics in the following way:

$$R_0 \propto \kappa^2 Q_D \int \mathrm{d}\lambda\, \lambda^4 F_D(\lambda) A_A(\lambda).$$

In this expression $F_D(\lambda)$ is the normalized emission spectrum of the donor as a function of the wavelength, λ, while $A_A(\lambda)$ is the absorption spectrum of the acceptor. Q_D is the fluorescence quantum yield of the donor. κ^2 is the so-called orientational factor, which depends on the relative orientation of the donor and acceptor transition dipole moments and may take any value between 0 and 4.

The strong dependence on distance makes FRET an optimal method to determine intramolecular distances in biological molecules [106]. The useful range for FRET with the dyes that are being typically used for single-molecule experiments is 20–100 Å. An accurate determination of the Förster distance for a particular donor–acceptor pair is required in order to enable distance measurements, and the largest uncertainty is due to the difficulty in estimating the orientational factor. It can be shown though that, if the donor and acceptor reorient on a timescale faster than τ, then an averaged value of $2/3$ can be used [107]. This averaged value is broadly used in the literature, although its application has to be justified in each case separately, as it depends crucially on the freedom of motion of the two fluorescent molecules. Schemes have been developed that allow putting limits on the possible error arising from the use of the averaged κ^2 in cases where the orientational motion of the donor and acceptor is limited. These schemes rely on the values of the fluorescence anisotropy of the fluorophores. The interested reader is referred to the original literature [108, 109].

k_{ET} can be measured in a time-resolved fluorescence experiment by measuring the change in the donor lifetime:

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_{ET}$$

where τ_0 is the donor lifetime in the absence of FRET. Under steady state illumination conditions one measures the FRET efficiency:

$$E_{ET} = \frac{R_0^6}{R^6 + R_0^6}$$

which can be obtained from the reduction in donor fluorescence intensity in the presence of the acceptor, from the increase in acceptor fluorescence intensity in the presence of the donor or, the best, by taking into account both intensities in the following manner:

$$E_{ET} = \frac{I_A}{I_A + I_D}$$

where I_A and I_D are the donor and acceptor intensities. This expression should be corrected for differences in donor and acceptor quantum yields as well as differences in the detection efficiencies at the respective wavelengths [74, 110].

If the distance between the donor and acceptor is distributed, e.g. due to conformational dynamics in a large biomolecule, an analysis of the type described above provides only the average FRET efficiency. Haas and co-workers [111] devised a method to obtain a parametrized distance distribution from time-resolved fluorescence experiments. This method has been extensively applied to study intramolecular dynamics, including protein folding [112, 113]. Single-molecule experiments provide a more direct way to obtain the FRET efficiency distribution. The FRET efficiency can be calculated from each burst in a diffusion experiment or from each point in a single-molecule trajectory obtained from an immobilized molecule. A histogram is then constructed from the series of values obtained in this way. By calculating the distance from each FRET efficiency value it is possible to construct also the distance distribution. Naturally, one is interested in broadening of this distribution caused by static or dynamic variations in the donor–acceptor distance, which can be related to structural and functional properties of a biomolecule under study. However, several other, spurious factors can also broaden this distribution.

First and foremost among these factors is the Poissonian photon noise in each of the channels in the experiment. The contribution of photon noise to the variance of the FRET efficiency distribution is given approximately by

$$\sigma_E^2 = \frac{E(1-E)}{N}$$

where E is the mean value of the FRET efficiency and N is the average total number of photons per point (burst). With 20 photons per point the contribution of photon noise to the full width half-maximum of a distribution with a mean FRET efficiency of 0.5 can be as large as 0.25.

Other factors that contribute to the FRET efficiency distribution are usually not as simple to quantify, but can sometimes be controlled and minimized. For example, the relative orientation of the donor and acceptor molecules might be distributed if the reorientational motion of the two probes is not fast enough to completely average it out. This can happen in cases where either the donor or acceptor interacts strongly with the macromolecule to which it is attached. Usually such strong interactions, which limit significantly the reorientational motion of fluorescent probe, can be deduced from fluorescence anisotropy measurements.

Another factor that may be distributed in single-molecule experiments is the quantum yield of the donor, which determines in turn the Förster distance. In some cases the quantum yield of the donor depends on the conformational state of the macromolecule. This can happen if a local interaction exists that tends to intermittently quench the donor fluorescence. If the rate of transition between a quenched and an unquenched state is slow on the timescale of a single-molecule experiment then it will lead to a distribution of Förster distances, and

therefore a further broadening of the distribution of FRET efficiency. Measurements of the fluorescence lifetime on a molecule-by-molecule basis can facilitate detection of this phenomenon [49].

A few words are in place here regarding the choice of fluorophores for single-molecule FRET studies and labelling techniques. Naturally, the first and foremost consideration for choosing a pair of fluorophores for FRET is matching their Förster distance to the average distance to be probed. Another important consideration is the photostability of the fluorophores, which is a key issue in single-molecule experiments, as it determines the time window available for data collection on each molecule. Finally, the water solubility of the fluorophores will dictate their interaction with the protein once they are attached to it and will determine how well their orientation is averaged by fast rotational motion, validating the use of an averaged orientational factor in FRET distance calculations.

There are several families of fluorophores which can be used for fluorescence spectroscopy of single molecules in general and single-molecule FRET in particular. These are usually dyes which have their lowest energy transition in the visible range of the spectrum, where it is most convenient to perform the types of experiments discussed above. Other common features of these dyes are quantum yields close to unity, and in some cases also good water solubility, obtained by the introduction of charged moieties such as sulfonate groups. Of these I will mention here rhodamine-like fluorophores and the families of Alexa dyes (molecular probes) and Cy dyes (Amersham). All of these fluorophores show reasonable photostability, which allows the extracting of many thousands of photons from them before photobleaching. It is possible in some cases to increase photostability by using triplet quenchers or oxygen scavengers, both lowering the propensity of a fluorophore to undergo excited-state reactions. A recent work shows that the application of oxygen scavenging enzymes can increase the number of photons measured from a single fluorophore to more than a million [114].

The problem of specific chemical labelling of a biomolecule with two fluorescent probes does not have a universal solution. This problem is simplified in the case of nucleic acids, since separate oligonucleotide chains can be labelled and then hybridized together [115]. Techniques of a similar spirit can be devised for proteins as well, especially if they are not too large. For example, Jia *et al* [74] synthesized and labelled separately the two helices of the GCN4 peptide they used in their single-molecule folding study, then ligated them together with a disulfide bond. In fact, peptide synthesis offers several options for the simplification of double labelling schemes [116, 117]. Such techniques cannot be applied to larger proteins that cannot be chemically synthesized. Haas and co-workers [118] have devised a multi-step process for specific labelling with two fluorophores, and have applied this process over the years to several different proteins [113, 119–121]. Their process relies on a careful application of chemical separation techniques to first prepare populations of protein molecules which are specifically labelled with a fluorophore at a unique position on the protein, typically a cysteine residue [118]. In a subsequent step a second residue is labelled with a different fluorophore. In some cases this general method can be simplified by the use of an N-terminal cysteine residue which can be labelled very specifically with a thioester-containing fluorescent probe [122].

It should be noted that proteins can also be labelled with genetically encoded fluorophores, i.e. fluorescent proteins [123]. A FRET pair can be obtained by attaching two different fluorescent proteins to the same biomolecule [124]. However, this approach does not seem to be so useful for folding studies, not only because of the large size of the probes, but also because they might be severely affected by the denaturing or partially denaturing conditions of such experiments. Another new type of fluorescent marker, semiconductor nanocrystals [125], will no doubt find useful applications in single-biomolecule spectroscopy, especially due to their high photostability.

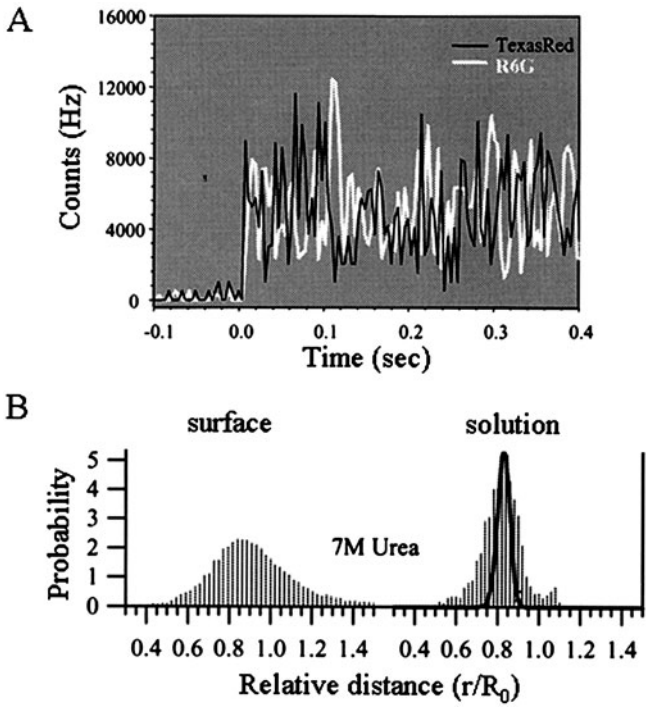

Figure 5. (A) Trajectory of a single GCN4 peptide, showing anti-correlated changes in the donor (R6G) and acceptor (Texas Red) fluorescence intensities, indicative of conformational changes due to folding and unfolding of the peptide. (B) Interdye distance distributions calculated from trajectories taken on surface-immobilized molecules (left) and from freely diffusing molecules (right) in the presence of 7 M urea. The surface distribution is much broader than the solution distribution. This effect was attributed to the interaction of the peptide with the surface (adapted with permission from [84]).

4. Single-molecule folding

The rather small but exciting body of published work on the folding of individual protein and RNA molecules will be described in this section in an essentially chronological fashion. I will also try to point to some generalities arising from the various studies.

4.1. Protein folding

Hochstrasser and co-workers [74, 84] pioneered single-molecule studies of protein folding with their investigation of the folding dynamics of a peptide prepared from the yeast transcription factor GCN4, which forms a cooperatively folded helical dimer. A version of the peptide, in which the two helices are connected by a disulfide bond, was prepared. One of the helices was labelled at its N-terminus with rhodamine 6G as a FRET donor, while the other helix was labelled with Texas Red as a FRET acceptor. The rate of folding of the labelled dimer was measured to be 150 s^{-1}. A negatively charged stretch of amino acids was inserted at the C-terminus of each helix to facilitate oriented attachment to an aminosylanized glass surface. FRET trajectories of single molecules were collected with a confocal microscope. Some of the trajectories showed fluctuations in FRET efficiency, which were attributed to folding/unfolding dynamics of the dimer (figure 5(A)). Correlation analysis showed that the timescale of these

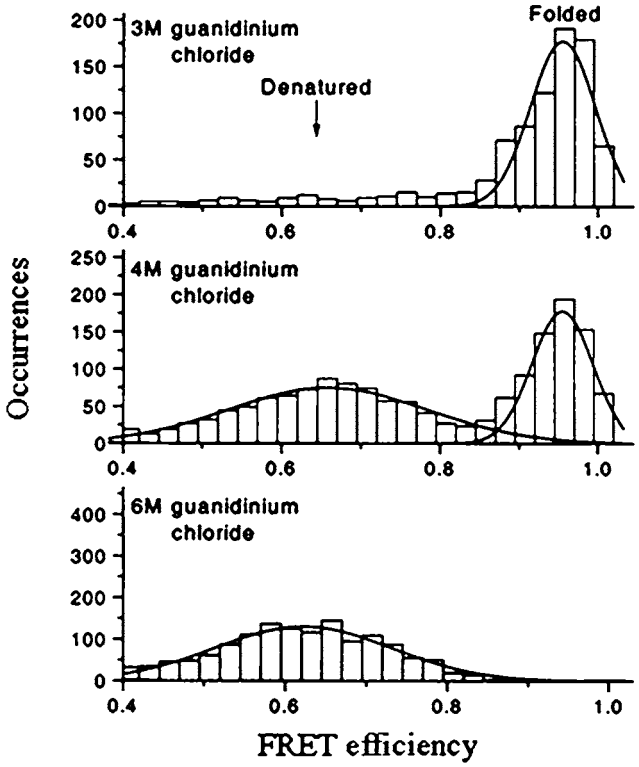

Figure 6. FRET efficiency distributions of chymotrypsin inhibitor molecules at three different concentrations of the denaturant guanidine hydrochloride, showing clearly the two-state folding behaviour of this protein. The sub-population of denatured proteins increases with the denaturant concentration (adapted with permission from [117]).

fluctuations indeed matched the ensemble timescale. However, analysis of FRET efficiency distributions, especially in comparison to similar distributions obtained on freely diffusing molecules, showed that surface attachment significantly affects the distribution. This effect was especially significant at high urea concentration, and the distribution of surface-attached molecules was much broader than the distribution obtained in solution (figure 5(B)).

To circumvent this difficulty, several workers resorted to studies on freely diffusing molecules as a means to probe folding under the most natural conditions.

Deniz *et al* [117] studied molecules of the protein chymotrypsin inhibitor 2 (CI-2), a two-state folder well characterized by ensemble techniques [126]. Molecules of CI-2 were made from two peptides that corresponded to the N- and C-terminal parts of the protein, respectively, which were prepared using solid-phase peptide synthesis methodology. Ligation of the two peptides relied on their interaction to form a structure similar to the native structure of the protein. This technique facilitated specific labelling of the protein with tetramethylrhodamine as the donor molecule and Cy-5 as the acceptor. FRET efficiency distributions were obtained at a range of denaturant concentrations. The distributions nicely showed two sub-populations, one corresponding to the native conformation and one to the unfolded one (figure 6). The latter distribution was significantly broader than the former. The authors suggested that the width is dominated by shot noise. An important finding was the shift of the average position of the denatured sub-population distribution to larger values as the denaturant concentration was

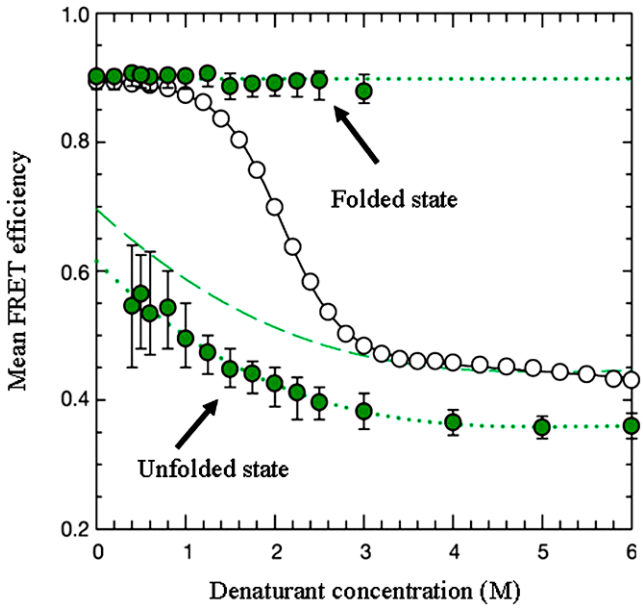

Figure 7. Dependence of the mean values of FRET efficiency distributions of the cold shock protein on the concentration of guanidine hydrochloride. The upper full symbols are from folded distributions while the lower full symbols are from unfolded distributions. Open symbols are ensemble FRET efficiencies. Note the significant shift of the mean of the unfolded population to higher values as the concentration of the denaturant decreases. This shift is attributed to the collapse of the protein chain (adapted with permission from [127]).

reduced. This shift could be attributed to the collapse of the denatured chain as the conditions become more and more native-like.

Schuler *et al* [127] chose a different two-state folder for their studies, a cold shock protein from the hyperthermophilic bacterium *Thermotoga maritima*. An engineered version of this protein was prepared with a cysteine residue at each of the termini, and these cysteine residues were labelled with the dyes Alexa 488 and Alexa 594. As in the CI-2 experiment, measurements on freely diffusing molecules were used to obtain FRET efficiency distributions under various concentrations of the chemical denaturant. These distributions also showed two sub-populations. The distribution of unfolded molecules showed an even more dramatic shift with denaturant concentration than seen in the CI-2 study, again attributed to chain collapse (figure 7). To understand the sources of broadening of FRET efficiency distributions, the authors performed measurements on several labelled polyproline peptides, considered to be rigid. They found that the width of the distribution from unfolded molecules is not larger than that of the polyproline peptide with the same average FRET efficiency value. They used this fact to put a lower limit on k_0, the rate of intrachain diffusion in the unfolded state of this protein, which has to be larger than 4×10^{-5} s^{-1} (an upper limit for this rate was already mentioned in section 1.1).

Two important new insights arise from the diffusion experiments described above. First, these experiments allow probing the collapse of a denatured protein as it is transferred from a good solvent to a bad solvent. While this collapse can be studied in fast kinetic experiments (see, e.g., [128]), single-molecule experiments allow the observation of partially collapsed states of the protein under equilibrium with the native state, and open the way to a deeper investigation of the structure of these intermediate states. Second, some information on internal dynamics

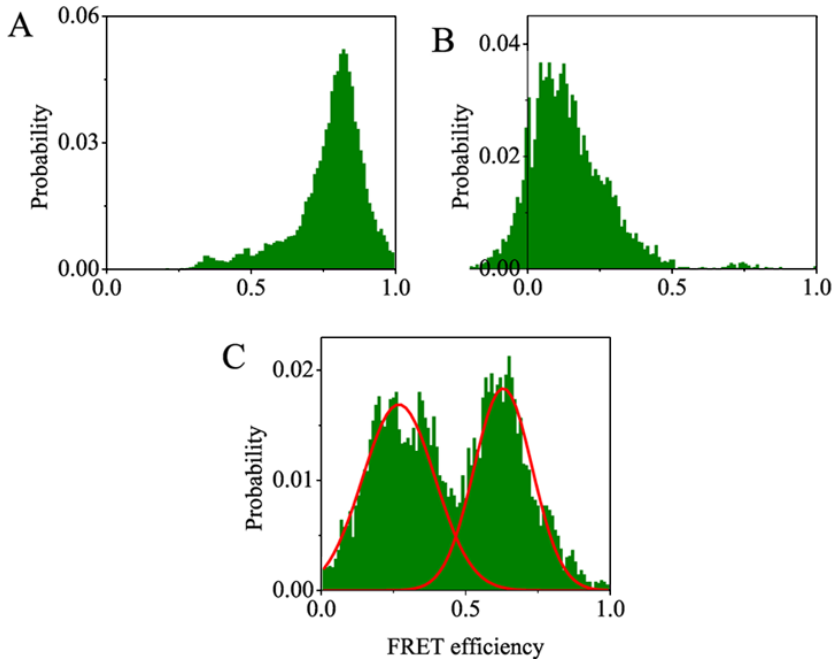

Figure 8. Distributions of FRET efficiency values obtained from single-molecule trajectories of labelled AK molecules trapped in vesicles. (A) Native conditions. (B) Denaturing (2 M GuHCl) conditions. The average values, 0.8 and 0.14, respectively, are close to the ensemble values. (C) Mid-transition conditions (0.4 M GuHCl). This distribution can be roughly divided into two sub-distributions, one due to the 'denatured' ensemble, with E_{ET} values smaller than ∼0.45, and one due to the 'folded' ensemble, with E_{ET} values larger than that value, as illustrated by the red (light grey) lines, which are Gaussian fits (from [88]).

can be obtained from such experiments, even though they do not directly probe conformational dynamics. Can more direct dynamic information be obtained from experiments on diffusing molecules? In principle, this should be possible if the residence time of single molecules in the laser beam can be increased. Such an increase can be achieved using viscogenic reagents, although care should be taken to avoid the effect of such reagents on the stability of the protein studied. The best way to obtain dynamic information on single folding molecules is clearly by immobilizing them on a surface.

A first step towards this goal was made by studying protein molecules trapped within surface-tethered lipid vesicles [71]. The 214-amino acid protein adenylate kinase was used in this study, and was labelled with Alexa 488 as the donor and Texas Red as the acceptor, which were specifically attached to cysteine residues inserted into the polypeptide chain. The FRET efficiency distributions obtained from trajectories of trapped molecules under native or fully denatured conditions match the ensemble-measured values (figures 8(A), (B)). At the midpoint of the denaturation curve the distribution is clearly bimodal, indicating the existence of one dominant free energy barrier under these conditions (figure 8(C)). This barrier separates two sub-populations of the protein, even though adenylate kinase is certainly not a two-state folder. The peak values of the two sub-distributions are shifted from the corresponding values in the fully folded or fully denatured distributions. The shift of the denatured distribution is again an indication of chain collapse due to a change in solvent conditions. The shift in the 'folded' distribution suggests that partially open conformations of the protein are populated under mid-transition conditions.

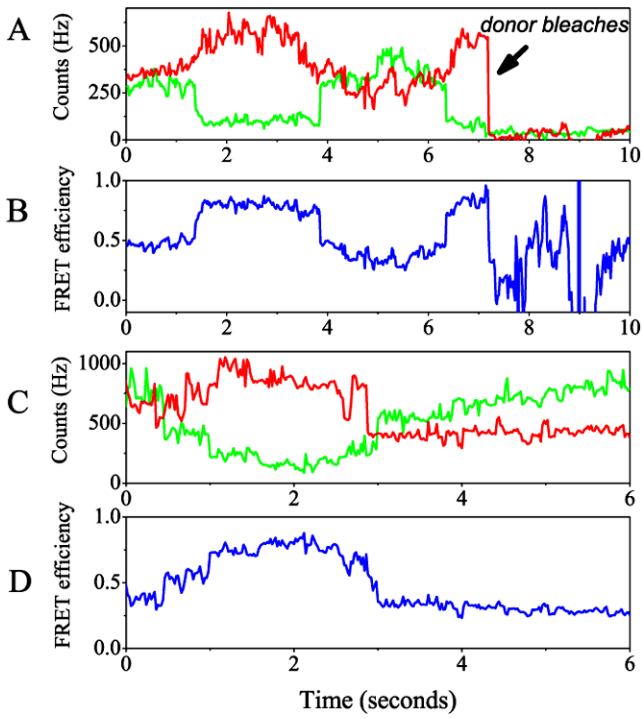

Figure 9. (A) Time traces of an individual vesicle-trapped AK molecule under mid-transition conditions, with the acceptor signal in red (black) and the donor in green (grey). The traces were collected with 20 ms time bins and smoothed using the forward–backward non-linear filter described in the text [91]. (B) FRET efficiency (E_{ET}) trajectory calculated from the signals in (A), (C) and (D). A single-molecule trajectory showing a slow transition between two states of the molecule, starting at ∼0.5 s and ending at ∼2 s. Donor and acceptor time traces in (C), FRET efficiency trajectory in (D) from [88].

The distribution is broadened by transitions between these substates of the protein, as can be verified by examining the trajectories themselves. Indeed, the trajectories show that some of these transitions transfer a molecule over the large free energy barrier mentioned above, while other transitions maintain the molecule within the same side of the barrier. Sample trajectories are shown in figure 9. Panels (A) and (B) show a trajectory with several jumps between a fully folded state of the protein (FRET efficiency of ∼0.8) and a partially folded state (FRET efficiency of ∼0.5). Panels (C) and (D) show an example of a 'slow transition', a transition between two states of the protein which does not occur instantaneously on the timescale of the measurement, as opposed to the transitions shown in (A) and (B). Such a slow transition, the like of which is seen quite frequently in the AK trajectories, actually allows tracing the conformation of the molecule as it changes from one state to another. It is hard to imagine that it can be due to a barrier crossing event, though, and a molecular picture of the origin of such slow motion on the energy landscape is still missing. One possibility is that it arises from very slowed-down intrachain diffusion experienced by this large protein on certain regions of its energy landscape.

Figure 10 shows a two-dimensional map of transitions seen in the trajectories, with the initial FRET efficiency value (i.e. the value before the transition) on the ordinate and the final value (after the transition) on the abscissa. The large dispersion of the points on this map

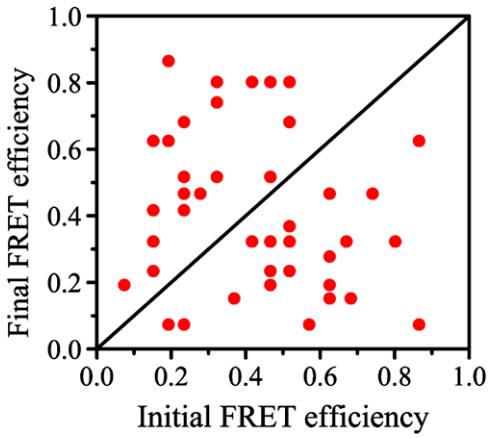

Figure 10. Map of folding/unfolding transitions obtained from single-molecule trajectories. Each point represents the final versus initial FRET efficiency for one transition. The line is drawn to distinguish folding and unfolding transitions: above the line are folding transitions (efficiency increases), while below the line are unfolding transitions (efficiency decreases) from [88].

shows that the dynamics of the protein are very heterogeneous. Further, the distribution of transition sizes (not shown) suggests that these dynamics involve motion between neighbouring intermediate states rather than large jumps between very different conformations. This picture is as expected for a rugged energy landscape, on which many local traps and barriers dictate the motion [9].

Exposing the heterogeneity of folding pathways and the rates of chain dynamics at various positions on the energy landscape is an important goal of single-molecule folding studies. The experiments discussed in this section have provided some interesting initial observations on this subject. Further confirmation for the energy landscape picture comes from studies of RNA folding.

4.2. RNA folding

Several aspects of RNA folding were studied extensively on the single-molecule level by the Chu and Herschlag groups. Particularly rich information was obtained in a series of studies on the *Tetrahymena* ribozyme [87, 129, 130]. Individual molecules of the ribozyme, labelled for FRET, were immobilized on a surface using biotin–streptavidin chemistry. Importantly, the authors were able to show that the ribozyme molecules are fully active when bound to the surface [87]. Further, a comparison of the overall folding yield to the yield obtained in ensemble studies showed that the surface does not significantly perturb the reaction. This should be contrasted with the strong surface influence on proteins discussed above.

The overall folding reaction was followed in a non-equilibrium experiment in which magnesium ions were added in order to initiate the reaction (figure 11(A)) [87]. Interestingly, two folding rates were identified from this histogram: a slow rate similar to that obtained from ensemble studies (0.016 s^{-1}), and a second, faster rate (1 s^{-1}). It was suggested that the occurrence of two rates is evidence for kinetic partitioning in the folding process [42]. The same experimental approach was later used in order to obtain a detailed picture of the folding energy landscape of the *Tetrahymena* ribozyme [129]. The folding reaction was initiated from various positions on the energy landscape by varying the concentration of sodium ions in the denaturation buffer. It was shown that different folding pathways can be accessed in this way.

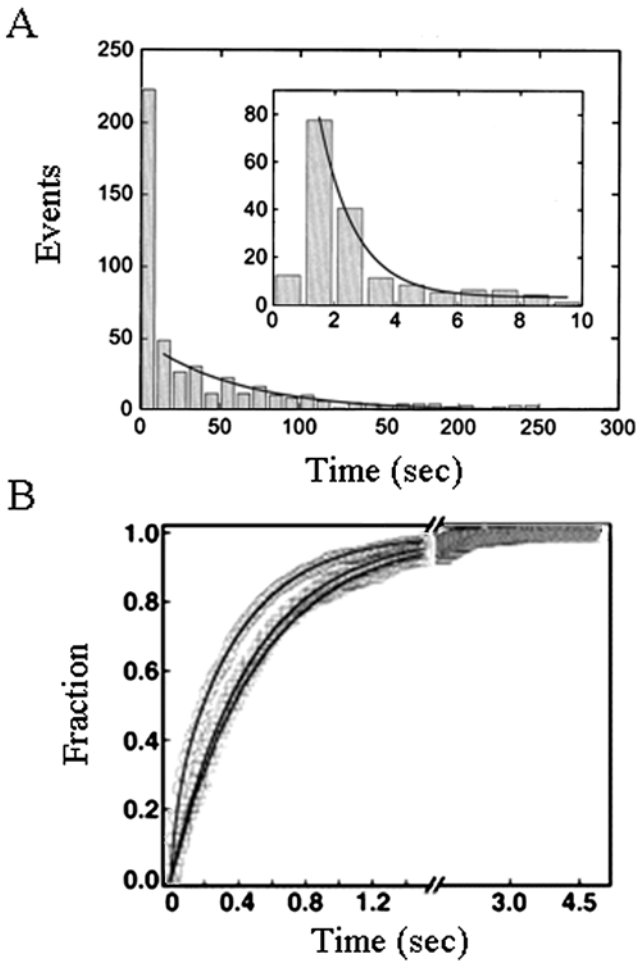

Figure 11. (A) Histogram of folding times obtained from a non-equilibrium experiment on the *Tetrahymena* ribozyme. Two folding times are obtained, the faster one shown in the inset (adapted from [87]). (B) A cumulative plot of time spent by the P1 helix in the undocked state, obtained from equilibrium trajectories. Curves are presented for the wild type and two modified helices. The modifications do not significantly alter the docking rate, indicating that the modified site is not involved in the folding transition state. A series of such experiments allowed Bartley *et al* [130] to conclude that the transition state of the docking reaction involves a kinetically trapped state of the ribozyme (adapted from [130]).

A more local folding event, involving docking of the P1 duplex into the preformed core of the ribozyme, was also characterized by the same group [87, 130]. A detailed ϕ-value analysis was performed by modifying groups on the duplex that make contact with the core of the rybozyme (figure 11(B)) [130]. This analysis suggested that the tertiary interactions involved in the docking reaction are not formed in the transition state [130]. Together with the slow docking rate this has led the authors to the conclusion that an escape from a kinetic trap is the rate-limiting step in docking.

Kim *et al* [55] used correlation analysis to study folding dynamics of a much smaller RNA molecule, a three-helix junction. Immobilization of the molecules allowed extraction of rates on a broad timescale from microseconds to milliseconds. The effect of magnesium on folding

and unfolding rates was interpreted as an indication for a significant stabilization of the folded state by ion binding.

These studies reaffirm the picture of RNA folding as a process occurring on a rough energy landscape. Slow rates, even for rather local folding events like the P1 duplex docking, are due to traps on the energy surface. Further, the initial conditions can be used to decide which of several pathways, separated from each other by large energy barriers, are accessed during the folding reaction.

5. Prospects

As is evident from the discussion above, single-molecule fluorescence spectroscopy is only starting to contribute to our understanding of biomolecular folding. Here I would like to outline some of the questions that will be of interest for this field in the coming years and suggest where it might make its largest contributions.

First, as discussed in section 1.1, the opportunity to probe the unfolded state under native conditions with ensemble techniques is rare, and in most proteins one has to be content with the availability of data on the structure under denaturing conditions. On the other hand, single-molecule experiments are able to separate those molecules that are denatured from those that are folded, or to probe one molecule as it undergoes transitions between these states. Thus it should be possible to directly probe dynamics in the unfolded state of a protein, identify the appearance of fleeting structure elements and measure the rates of their formation and disappearance. An interesting recent suggestion, based on molecular dynamics simulations of small, denatured proteins under native conditions, is that, while each molecule retains a random conformation, the average topology is similar to the native state [131]. To verify this assertion experimentally one needs to build the average structure from its constituents, a natural exercise for single-molecule studies. As it is expected that dynamics in the unfolded state will be rather fast, one will have to rely on techniques such as those developed by Xie and co-workers [56], in which a correlation analysis is carried out on a photon-by-photon basis. It is possible that other modalities of fluorescence spectroscopy other than FRET will be useful here, in particular polarization spectroscopy, that might pick up changes in segmental rigidity or transient formation of secondary-structure elements.

Second, it is highly desirable to develop the ability to directly detect multiple folding pathways and a broad distribution of substates. This ability should allow us to more fully characterize the free energy landscape of the folding molecule and characterize its level of ruggedness. Technically, the possibility of identifying multiple pathways, as has already been done in the study of Rhoades *et al* [88], relies on a rather slow interconversion between intermediate states formed on the way to folding. It is feasible that in small proteins, which show two-state folding behaviour on the ensemble level, the rate of interconversion between various substates in the ensemble of structures describing the folded or unfolded states will be too fast for direct probing. This rate can, in principle, be slowed down using viscogenic reagents, with the caveat that these chemicals might also affect the overall stability of a protein [132].

Another important issue related to folding pathways is the ability to probe the actual transition between folded and unfolded states or, in other words, to resolve the motion of a molecule over the folding barrier. While methods like ϕ-value analysis provide indirect information about the structure of the transition state, it will definitely be of great interest to directly probe this structure, and this is not generally possible with ensemble kinetic methods. Again, a judicious application of viscogenic reagents can lead to the slowing down of the folding dynamics to the extent that motion over the folding barrier can be directly probed.

Finally, some general statistical mechanical aspects of the folding dynamics of individual biomolecules are also worthwhile studying. For example, the question of the approach to ergodic behaviour [133] is quite intriguing, and might shed some more light on the rate of sampling of substates, as well as on the accessibility of different regions on the energy landscape. Short-time deviations from ergodic behaviour are commonly observed in disordered systems, especially close to the glass transition. Single-molecule experiments have already shown deviations from ergodicity in the conformational dynamics of DNA molecules [134] and enzymes [64, 135].

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