Published in final edited form as:

J Phys Chem Lett. ; 4(16): 2727–2733. doi:10.1021/jz401259y.

Single-Molecule Analysis of Cytochrome *c* Folding by Monitoring the Lifetime of an Attached Fluorescent Probe

Andrea J. Lee[#], Wesley B. Asher[#], Harry A. Stern, Kara L. Bren^{*}, and Todd D. Krauss^{*}
Department of Chemistry, University of Rochester, Rochester, NY 14627-0216, United States

[#] These authors contributed equally to this work.

Abstract

Conformational dynamics of proteins are important for function. However, obtaining information about specific conformations is difficult for samples displaying heterogeneity. Here, time-resolved fluorescence resonance energy transfer is used to characterize the folding of single cytochrome *c* molecules. In particular, measurements of the fluorescence lifetimes of individual cytochrome *c* molecules labeled with a single dye that is quenched by energy transfer to the heme were used to monitor conformational transitions of the protein under partially denaturing conditions. These studies indicate significantly more conformational heterogeneity than has been described previously. Importantly, the use of a purified singly-labeled sample made a direct comparison to ensemble data possible. The distribution of lifetimes of single-proteins was compared to the distribution of lifetimes determined from analysis of ensemble lifetime fluorescence data. The results show broad agreement between single-molecule and ensemble data, with a similar range of lifetimes. However, the single-molecule data reveal greater conformational heterogeneity.

Keywords

confocal microscopy; change-point algorithm; single-molecule fluorescence lifetime; protein folding

Conformational dynamics of proteins, ranging from vibrations to large-scale folding, are important for function.¹ However, investigating dynamics is challenging, due to the inherent conformational heterogeneity of proteins.² Previously, single molecule fluorescence resonance energy transfer (smFRET) methods have been employed to examine protein folding.^{3–6} Unlike ensemble methods, smFRET gives information about conformations sampled by individual molecules while traversing the folding-energy landscape. The application of smFRET to protein folding has yielded many notable findings, including the earliest observations of transitions between folded and unfolded states,⁷ the characterization of entropic folding in a slow folder,⁸ the measurement of the time scale of fast folding transitions^{9,10} and the observation of multiple conformational states of a large protein.¹¹ Studies of intrinsically unfolded and denatured proteins have revealed slow dynamics in

^{*}Corresponding Author: bren@chem.rochester.edu; krauss@chem.rochester.edu.

Author Contributions

W.B.A. cloned, expressed, purified, and fluorescently labeled K99C biotin-tagged cyt *c*. Single molecule experiments were designed by W.B.A., A.J.L., T.D.K., and K.L.B., carried out by A.J.L. and W.B.A., and analyzed by A.J.L., W.B.A., and H. A. S. H. A. S. programmed the software used to analyze single molecule data. Ensemble experiments were carried out and analyzed by W.B.A. All authors contributed to writing the manuscript. K.L.B. and T.D.K. conceived the work.

SUPPORTING INFORMATION

Supporting Information for publication. Materials and Methods. Supporting Figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

intrinsically disordered proteins,¹² and determined reconfiguration times and internal friction for unfolded proteins and folding transition states.^{13,14} In addition to fundamental studies of folding, analysis of conformational changes related to function have benefited from smFRET approaches.¹⁵ Further advances in the field will require multidimensional energy landscapes to be constructed from experimental data on large and complex proteins over long time scales.¹¹ In addition, comparisons between experimental single-molecule and ensemble data are needed to facilitate the use of ensemble control experiments and to better relate results obtained on the single molecule level to literature data on similar systems, the preponderance of which is obtained on the ensemble level.

Horse cytochrome *c* (cyt *c*) is an ideal system for probing structural complexity using smFRET for several reasons. First, the folding of cyt *c* on the ensemble level has been characterized with a variety of techniques, providing important benchmarks for single molecule studies.^{16–19} For example, ensemble studies at equilibrium reveal a folding intermediate with characteristics broadly consistent with the N- and C-terminal helices being formed and docked, i.e., similar to the on-pathway kinetic intermediate.^{16,20,21} Also, NMR studies have shown that changes in loop 3 observed in the folding intermediate populated in denaturant are similar to those seen at high pH (i.e. the alkaline transition).²² Second, ensemble FRET has also been used to study cyt *c* folding, including detection of an intermediate,^{23–25} allowing for direct comparisons to smFRET data. Finally, the significance of cyt *c* conformational dynamics is highlighted by the identification of conformational changes of cyt *c* linked to its role in apoptosis.²⁶

It is common in smFRET studies of protein folding to use the intensities of two fluorescent dyes, a donor and acceptor, to track FRET efficiency as the protein conformation changes.^{3,4,6} The lifetime of the donor in the presence (τ_{DA}) and absence (τ_D) of the acceptor is related to the FRET efficiency ($E = 1 - (\tau_{DA}/\tau_D)$), which depends on the donor-acceptor distance. An advantage of using lifetimes is that, unlike FRET studies based on detecting fluorescence intensities, lifetimes are not affected by variation of experimental conditions such as laser focus or intensity. This donor-lifetime approach has been applied to study the dynamics of a disordered protein to which two dyes have been attached.¹³ For cyt *c*, the covalently bound heme can be used as a FRET acceptor for a single chemically attached donor dye.^{23–25} Thus the dye-heme proximity can be probed by a single-color FRET measurement during folding. In the folded protein, the donor fluorescence is quenched by its close proximity to the heme (Figure 1a), resulting in a short fluorescence lifetime. In the presence of denaturant, the protein unfolds and the donor lifetime increases.

In this study, we use single-color, time-resolved smFRET to study the folding of horse cyt *c*. Typically, smFRET studies utilize proteins labeled with two extrinsic dyes, a donor and an acceptor. However, it is extremely difficult to prepare pure samples of proteins labeled with two dyes, and a highly purified sample is a requirement for ensemble studies of folding. Herein, we use an extrinsic dye as a donor and the intrinsic heme as the acceptor. Using one dye facilitates site-specific labeling and enables preparation of samples in pure form, allowing direct comparison of lifetime distributions from SM and ensemble experiments.²³ Thus, for the typical smFRET studies that use proteins labeled with two dyes, a direct comparison to an ensemble experiment is not possible. The use of a time-resolved FRET approach also allows for mitigation of non-FRET related quenching processes that can cause donor intensity variations,^{14,15} thus providing a relatively simple determination of FRET efficiencies.

Confocal microscopy and time-correlated single photon counting (TCSPC) methods (supplementary information, SI) were used to monitor the fluorescence lifetime of single Alexa Fluor® 488-labeled horse heart K99C-Fe(III) cyt *c* (K99C-AF488) (Figure 1). The

protein was tagged with a biotin-containing fusion peptide (biotin tag) and immobilized on a polyethylene glycol (PEG) – biotin quartz surface by avidin-biotin chemistry (SI) to observe the molecules over an extended period of time (Figure 1a). This particular immobilization technique prevents proteins from interacting with the surface.⁹ Using a surface tethering approach enables long observation times (~10 s), whereas experiments that are conducted on molecules as they freely diffuse through a confocal laser volume are limited in observation time to ~1 ms..³

We exposed immobilized K99C-AF488 samples to the denaturant guanidine hydrochloride (GuHCl) at concentrations near the folding transition midpoint (1.75, 2.00, 2.25, 2.50 M GuHCl; SI Figure S1), at which the protein conformation is likely to fluctuate. GuHCl, even at concentrations below that needed to induce complete protein unfolding, promotes the population of non-native conformational states, and has been widely used in studies of non-native conformations of proteins including cyt *c*.^{16,21,22,24} Images of K99C-AF488 at 0 M GuHCl show only 1–3 bright spots on average per image (Figure 1b), consistent with a sample containing mostly folded proteins with a high FRET efficiency and thus almost fully quenched donors. An increase in number of unquenched molecules appears at 1.75 M GuHCl, while at 2.5 M, the AF488 donors are brighter, indicating decrease of FRET as would result from unfolding (Figure 1b). Images of PEG-biotin surfaces treated with K99C-AF488 in the absence of bound neutravidin and at 2 M GuHCl show minimal immobilization of the protein, confirming that the increase in brightness with increasing GuHCl concentration is a result of unfolding of proteins tethered to the surface (SI Figure S3).

To monitor the folding transitions of individual molecules, we collected TCSPC data to generate intensity and lifetime trajectories (Figure 2). We use the intensity state-finding change-point algorithm²⁷ to reconstruct fluorescence intensity vs. time plots. The change-point algorithm does not require that photons are binned into time intervals before processing, thus eliminating errors from averaging that can occur from binning all photon arrival times. Using simply the photon arrival time statistics, the change point algorithm assigns individual photons to different intensity states and thus eliminates bias associated with finding these states (Figure 2a). After processing by change-point, the arrival times of photons in each change point-derived intensity state were binned (Figure 2b). Next, the convolution of a single exponential and the measured instrument response function were fit to the resulting histogram, using a maximum likelihood estimator (MLE) method²⁸ (Figure 2b) to determine the lifetime of each state (Figure 2c).

The sensitivity and time resolution in a time trace are limited by the total photon counts in each intensity state. The change-point algorithm allows a time resolution on the order of 100 ms for the lowest intensity states of the donor dye under our observation. However, we analyzed only those intensity states with > 200 total photons to ensure a reasonably small error (13%) in the MLE calculated lifetimes (see SI). Thus, our smFRET measurements are not expected to observe fast folding transitions, which have been reported to occur on the single microsecond scale in single-molecule folding experiments.¹⁰ Instead, our method is designed to detect the presence of relatively stable conformational intermediates that a single protein samples at equilibrium. Specifically, the cyt *c* smFRET folding trajectories show a range of states that persist for milliseconds to seconds (SI Figure S5). In addition, most trajectories show fluctuations among several states (SI Figure S5). We compiled hundreds of lifetime states from each denaturant condition for further analysis.

Population distributions of SM lifetime states show how the fluorescence lifetime of K99C-AF488 changes as a function of denaturant (Figure 3a). In these plots, lifetime states were binned with a resolution of 0.1 ns, and the relative occurrence of each binned state was

weighted by the dwell time of each particular state across all molecules. The SM histograms show evidence of multiple states, which change in lifetime and relative population as the concentration of denaturant is increased. At 1.75 M GuHCl, a highly quenched population (lifetime ~ 0.3 ns), consistent with the FRET efficiency expected for the folded conformation,²³ dominates the distribution. These quenched states are less populated at 2.50 M GuHCl. Interestingly, at all denaturant concentrations, the protein accesses fluorescence lifetime states that span a range from 0.3–2.9 ns. It is clear from the SM lifetime distributions that partially denatured cyt *c* is conformationally heterogeneous with many structural species populated. This level of detail has been hidden in previous ensemble studies of cyt *c* folding.

To ensure that our SM data is consistent with ensemble measurements, we compared TCSPC fluorescence decay curves constructed from the immobilized SMs with the ensemble decay curve from a 150 nM solution of freely diffusing K99C-AF488 collected on the same microscope under identical surface conditions (Figure 3b). The photons in the TCSPC decays for all of the states from the SM measurements were used to derive ensemble decays, referred to as the SM-derived ensemble. The decay curves for both the SM-derived ensemble and true ensemble are multiexponential, and overlap well, indicating that, as expected, an ensemble average of SM data reproduces the ensemble data. The broad agreement between the SM (tethered) and ensemble (freely-diffusing) data also indicate that tethering is not introducing a major perturbation to the folding of the protein, as the tethered single molecules observed are unlikely to be interacting significantly with the surface. The minor differences in overlap between the SM-derived and true solution ensemble data in Figure 3b at 2.25 and 2.5 M GuHCl may be attributed to differences between immobilized and diffusing proteins, and/or a higher likelihood of donor bleaching for unfolded versus folded proteins at higher denaturant concentrations for the SM samples, compared with the ensemble samples. In short, the similarities between the ensemble averaged SM data of tethered protein and ensemble fluorescence data on freely diffusing protein in solution, provide an important control regarding the validity of acquiring folding data on single tethered proteins.

The observed lifetimes in Figure 3a were also compared with previously reported values for cyt *c*. We determined lifetime values for individual K99C-AF488 of 0.3 ns for compact states consistent with folded protein and 2.6–2.7 ns for extended states consistent with unfolded protein. A lifetime of 0.3 ns indicates a FRET efficiency of 93%, which agrees with predictions of a dye-heme distance of ~ 29 Å for the native state of cyt *c*.²³ Furthermore, the unfolded state lifetime values of 2.6–2.7 ns agree with predicted lifetimes based on unfolded conformations obtained from ensemble fluorescence intensity-based FRET measurements of K99C-AF488.²³ Single molecule lifetimes corresponding to the unfolded state are shorter than would be predicted if the unfolded state of cyt *c* were a random coil.^{23,24} However, Zare and coworkers note that yeast cyt *c* never reaches a full unquenched state under fully denaturing conditions.²⁹ Other studies have suggested that the unfolded state of cyt *c* is not a random coil;²⁴ our results support those findings by showing shorter lifetime states than would be expected for a random coil. It is also possible, however, that the dynamics of the unfolded polypeptide are faster than the resolution of the measurement, and extended conformations are sampled at a rate that is too fast for detection using the current system.

Previous studies of cyt *c* folding using time-resolved ensemble FRET have typically used lifetime distribution models to analyze the multiexponential decays.^{24–26,30} For example, in time resolved FRET studies of yeast cyt *c* folding on the ensemble level,^{24,30} distributions of donor-acceptor distances (or, equivalently, FRET lifetimes) were extracted from the fluorescence decay curves using a maximum entropy method (MEM) algorithm.^{31,32} We fit

our ensemble decay curves to lifetime distributions using the MEMexp program (Figure 3c), which is a recent improvement to standard MEM methods. MEMexp is particularly well-suited for deriving lifetime distributions from multixponential decays and can resolve lifetime components that are not observed using MEM methods alone.³² Our approach gave similar distributions to those reported previously for ensemble yeast cyt *c* folding.^{24,30} This broad agreement provides further support for a lack of significant surface effects on folding in our experiments. A difference to note between the present data on horse cyt *c* and the literature data on yeast cyt *c* is in the denaturant concentrations needed to unfold the protein. As horse cyt *c* is significantly more stable, more denaturant is needed to promote the sampling of unfolded conformations.

We found the general trends in the SM distributions (Figure 3a) are consistent with the MEMexp distributions (Figure 3c); both show a proportional decrease in the population of compact states (~0.3-ns lifetime) and an increase population of in the unfolded state (2.6–2.7 ns) as denaturant concentration is increased. The change in the distribution from short- to long-lifetime states as concentration is increased is consistent with studies of yeast cyt *c* denaturation as well.^{24,30} Thus, we conclude that, as measured on either the SM level or the ensemble level, our overall distribution data is broadly consistent with published studies of cyt *c* folding, thus providing credence to the SM data and our method of acquiring it. However, using an SM approach, we can resolve lifetime states that can not possibly be observed in the MEMexp analysis of the ensemble data for cyt *c*. The ensemble distributions indicate two folding states, each of which contains a distribution of conformations. In contrast, the SM data reveal a broad range of conformational states extending from compact to extended, as is especially noticable for data collected for [GuHCl] between 2.0 and 2.5 M. Indeed, the SM results are in better agreement with studies that have identified a folding intermediate for cyt *c*; ^{21,22,33} no intermediate is revealed in the MEMexp analysis of the ensemble data. Differences in details observed between SM and ensemble should be expected since a major shortcoming of MEM programs (including MEMexp) is their difficulty in resolving lifetime distributions that are overlapping and broad, which is also related to the single-to-noise level of the decays.^{31,34} Consequently, it is possible that certain lifetime distribution components are not resolved, resulting in distributions that may not reveal all aspects of the heterogeneity sampled along a folding pathway. Thus, while direct comparison between MEMexp and SM lifetime distributions shows that similarities do exist, the ensemble lifetime distribution approach cannot reveal the level of detail needed to characterize the heterogeneous ensemble of cyt *c* conformations.

A notable feature of the SM trajectories for cyt *c* is the long lifetimes seen for extended states. The presence of stable structural intermediates in cyt *c* SM folding trajectories is not unexpected. Haran and coworkers have shown that under equilibrium conditions at the midpoint of folding, unfolded states are stable for seconds in a two-state folder (cold shock protein from *Thermotoga maritima*),³⁵ and unfolded and intermediate states are sampled for seconds for a multi-state folder (adenylate kinase).^{8,11} In addition, single molecule studies of proteins with folding events taking place on the order of microseconds nonetheless in the presence of denaturants show relatively long dwell times in unfolded states under equilibrium conditions.^{9,10,36} Furthermore, cyt *c* folding is known to be a complicated process with several confirmed intermediates and multiple possible folding paths. Englander and coworkers have proposed that substructural regions of cyt *c* are able to unfold independently of one another, and these substructures regularly sample unfolding conformations even under native conditions.¹⁶ Misligation of the heme cofactor also generates two stable intermediates.^{22,33} The fact that intermediate states are populated for perhaps seconds is not inconsistent with the fast folding times reported for cyt *c* previously based on measurements of intrachain diffusion times.^{37–39} The latter studies measure the time for a protein to change conformation, and not necessarily the time that it spends in that

conformation. In fact, the apparent discrepancy between the extremely fast times to change protein conformation ($< 1\mu\text{s}$), and the relatively slow folding times for the entire protein ($\sim\text{ms}$),⁴⁰ has recently been resolved,¹⁰ and shown to be completely consistent.

To examine the relationships between states detected and protein conformational fluctuations, the correlation between initial and final lifetime states for each analyzable transition in the SM time traces was plotted as a 2D histogram (Figure 4). These diagrams are symmetric about the $i = f$ line, which suggests that reversible conformational changes are observed in these experiments, and that the surface and/or the biotin-avidin attachment chemistry has a negligible effect on protein conformation. Chemical denaturant affects the folding landscape by changing the relative free energy of conformational states and thus altering energy barriers between states.⁴¹ We see clear evidence for these effects in Figure 4. At 1.75 M GuHCl, transitions between quenched and unquenched states are evident, with relatively few transitions among long lifetime states detected. As the denaturant concentration increases, the transitions are smaller in magnitude, and occur mainly among unquenched states, indicating that many extended conformations are energetically accessible. These results indicate that above the denaturation transition midpoint, the denatured state lies within a broad energy well that contains many unfolded conformational states of similar energies separated by energy barriers. These results are consistent with other single molecule studies of unfolded proteins.⁴²

Most studies of protein folding thus far using SM FRET have examined proteins that display 2-state behavior at equilibrium, with FRET efficiency (and thus distance) distributions representing the native and unfolded states.^{42–48} For example, *Thermotoga maritima* cold shock protein (CspTm) also displays two-state behavior on the ensemble and on the single-molecule level.⁴⁹ However, intermediates have been observed in SM studies of citrine,⁵⁰ Im9,⁵¹ and yeast cyt *c*,^{25,29,52} yet intermediates also are observed for these proteins in ensemble studies. We have found that cyt *c*, which exhibits more complex folding behavior than most proteins previously studied on the SM level, displays a rich heterogeneity of folding intermediates (with associated transitions between these intermediate states) not previously observed in ensemble studies.

Three previous studies of cyt *c* folding have been reported using smFRET methods (fluorescence correlation spectroscopy has also been employed but is not discussed here because it is sensitive to a much shorter time scale than the present studies). Perroud and coworkers used donor-labeled yeast-cyt *c* and the photon counting histogram model to probe the conformations of freely diffusing protein.²⁹ Kinoshita and coworkers developed methods to extend the observation time to $\sim 100\text{ ms}$ for probing freely diffusing dye-labeled yeast-cyt *c*.⁵² Although both studies showed evidence of structural complexity of cyt *c* folding, the time limitation imposed by studying freely diffusing molecules prevents transitions between conformational states from being investigated. One publication describing the folding of immobilized cyts *c* labeled with AF488 using a SM lifetime approach has been reported.⁵³ The authors immobilized cyts *c* in dry trehalose gels. We have found that trehalose does not provide a native-like environment for the protein, likely because the protein is not sufficiently hydrated.²³ Indeed, the authors of Ref. 53 detect a substantial signal for unfolded protein in the absence of denaturant, when the protein should present no such signal. In addition, the authors integrate over entire intensity time traces derived from a single molecule to generate ensemble-like TCSPC histograms, which are analyzed using the multiparameter correlation-prone NLLS fitting approach. All told, it is difficult to draw conclusions or make comparisons from this previous study that could be related to our data.

Our results reveal denaturant-dependent distance changes between a dye at residue 99 in the C-terminal helix of the protein and the heme attached to residues 14–18, which is a one-

dimensional view of the folding process. By using the covalently bound heme cofactor as the FRET acceptor, we can easily engineer several variants for donor labeling at multiple sites throughout the cyt *c* polypeptide chain.^{23,24} Thus, we can use our single molecule approach to collect rich information about structural changes that occur among the various folding substructures of the protein (forthcoming work). In addition, the methods described here can be used to study the folding (or conformational changes associated with protein function) of a range of proteins by genetically appending short fusion peptides containing covalently bound heme, known as heme tags, to the C- or N-termini of proteins.^{54,55} The smFRET methods employed here could find wide application for studying conformational changes or protein-protein interactions involving proteins that bind chromophores or metal ions.

In conclusion, we show that the fluorescence lifetime of a dye bound to a specific site on cyt *c* can be used to probe the folding of cyt *c* at the single molecule level. Using the methods employed here, we demonstrate that the true structural heterogeneity of partially denatured cyt *c* is more complex than previously reported. We anticipate that the approach described here will allow for a detailed multidimensional picture of the folding landscape for cyt *c*, in addition to providing valuable information necessary to better understand the complexities of protein conformational dynamics in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Peter Steinbach (National Institutes of Health) and Jan Ilavsky (Argonne National Laboratory) for their helpful discussions regarding MEM fitting and theory. We thank Marat Khafizov (Idaho National Laboratory) for programming raster scanning software in Labview used for data collection.

Funding Sources

This work was supported by the National Science Foundation (PHYS-0646565; CBET-0708599), the National Institutes of Health (GM63170, to KLB), and the Elon Huntington Hooker fellowship (to WBA).

REFERENCES

- (1). Grant BJ, Gorfe AA, McCammon JA. Large Conformational Changes in Proteins: Signaling and Other Functions. *Curr. Opin. Struct. Biol.* 2010; 20:142–147. [PubMed: 20060708]
- (2). Frauenfelder H, Sligar SG, Wolynes PG. The Energy Landscapes and Motions of Proteins. *Science.* 1991; 254:1598–1603. [PubMed: 1749933]
- (3). Ferreon ACM, Deniz AA. Protein Folding at Single-molecule Resolution. *Biochim. Biophys. Acta.* 2011; 1814:1021–1029. [PubMed: 21303706]
- (4). Schuler B, Eaton WA. Protein Folding Studied by Single-molecule FRET. *Curr. Opin. Struct. Biol.* 2008; 18:16–26. [PubMed: 18221865]
- (5). Chen HM, Rhoades E. Fluorescence Characterization of Denatured Proteins. *Curr. Opin. Struct. Biol.* 2008; 18:516–524. [PubMed: 18675353]
- (6). Schuler B, Hofmann H. Single-molecule Spectroscopy of Protein Folding Dynamics-Expanding Scope and Timescales. *Curr. Opin. Struct. Biol.* 2013; 23:36–47. [PubMed: 23312353]
- (7). 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.
- (8). Rhoades E, Gussakovskiy E, Haran G. Watching Proteins Fold one Molecule at a Time. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:3197–3202. [PubMed: 12612345]

- (9). 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. U.S.A.* 2009; 106:11837–11844. [PubMed: 19584244]
- (10). Chung HS, McHale K, Louis JM, Eaton WA. Single-molecule Fluorescence Experiments Determine Protein Folding Transition Path Times. *Science*. 2012; 335:981–984. [PubMed: 22363011]
- (11). 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. Comm.* 2011; 2:1–7.
- (12). Choi UB, McCann JJ, Weninger KR, Bowen ME. Beyond the Random Coil: Stochastic Conformational Switching in Intrinsically Disordered Proteins. *Structure*. 2011; 19:566–576. [PubMed: 21481779]
- (13). Soranno A, Buchli B, Nettels D, Cheng RR, Mueller-Spaeth S, 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. U.S.A.* 2012; 109:17800–17806. [PubMed: 22492978]
- (14). Borgia A, Wensley BG, Soranno A, Nettels D, Borgia MB, Hoffmann A, Pfeil SH, Lipman EA, Clarke J, Schuler B. Localizing Internal Friction Along the Reaction Coordinate of Protein Folding by Combining Ensemble and Single-Molecule Fluorescence Spectroscopy. *Nat. Comm.* 2012; 3:1–9.
- (15). Landes CF, Rambhadrar A, Taylor JN, Salatan F, Jayaraman V. Structural Landscape of Isolated Agonist-binding Domains from Single AMPA Receptors. *Nat. Chem. Biol.* 2011; 7:168–173. [PubMed: 21297640]
- (16). Bai YW, Sosnick TR, Mayne L, Englander SW. Protein Folding Intermediates - Native State Hydrogen Exchange. *Science*. 1995; 269:192–197. [PubMed: 7618079]
- (17). Weinkam P, Zimmermann J, Romesberg FE, Wolynes PG. The Folding Energy Landscape and Free Energy Excitations of Cytochrome *c*. *Acc. Chem. Res.* 2010; 43:652–660. [PubMed: 20143816]
- (18). Cherney MM, Bowler BE. Protein Dynamics and Function: Making New Strides with an Old Warhorse, the Alkaline Conformational Transition of Cytochrome *c*. *Coord. Chem. Rev.* 2011; 255:664–677.
- (19). Lyubovitsky JG, Gray HB, Winkler JR. Mapping the Cytochrome *c* Folding Landscape. *J. Am. Chem. Soc.* 2002; 124:5481–5485. [PubMed: 11996590]
- (20). Krishna MMG, Lin Y, Mayne L, Englander SW. Intimate View of a Kinetic Protein Folding Intermediate: Residue-resolved Structure, Interactions, Stability, Folding and Unfolding Rates, Homogeneity. *J. Mol. Biol.* 2003; 334:501–513. [PubMed: 14623190]
- (21). Latypov RF, Cheng H, Roder NA, Zhang J, Roder H. Structural Characterization of an Equilibrium Unfolding Intermediate in Cytochrome *c*. *J. Mol. Biol.* 2006; 357:1009–1025. [PubMed: 16473367]
- (22). Russell BS, Melenkivitz R, Bren KL. NMR Investigation of Ferricytochrome *c* Unfolding: Detection of an Equilibrium Unfolding Intermediate and Residual Structure in the Denatured State. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:8312–8317. [PubMed: 10880578]
- (23). Ensign AA, Jo I, Yildirim I, Krauss TD, Bren KL. Zinc Porphyrin: A Fluorescent Acceptor in Studies of Zn-cytochrome *c* Unfolding by Fluorescence Resonance Energy Transfer. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:10779–10784. [PubMed: 18669660]
- (24). Pletneva EV, Gray HB, Winkler JR. Many Faces of the Unfolded State: Conformational heterogeneity in denatured yeast cytochrome *c*. *J. Mol. Biol.* 2005; 345:855–867. [PubMed: 15588831]
- (25). Werner JH, Joggerst R, Dyer RB, Goodwin PM. A Two-dimensional View of the Folding Energy Landscape of Cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:11130–11135. [PubMed: 16844777]
- (26). Hanske J, Toffey JR, Morenz AM, Bonilla AJ, Schiavoni KH, Pletneva EV. Conformational Properties of Cardiolipin-bound Cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109:125–130. [PubMed: 22190488]

- (27). Watkins LP, Yang H. Detection of Intensity Change Points in Time-resolved Single-molecule Measurements. *J. Phys. Chem. B.* 2005; 109:617–628. [PubMed: 16851054]
- (28). Edel JB, Eid JS, Meller A. Accurate Single-molecule FRET Efficiency Determination for Surface-immobilized DNA Using Maximum Likelihood Calculated Lifetimes. *J. Phys. Chem. B.* 2007; 111:2986–2990. [PubMed: 17388421]
- (29). Perroud TD, Bokoch MP, Zare RN. Cytochrome *c* Conformations Resolved by the Photon Counting Histogram: Watching the Alkaline Transition with Single-molecule Sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:17570–17575. [PubMed: 16314563]
- (30). Pletneva EV, Gray HB, Winkler JR. Snapshots of Cytochrome *c* Folding. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:18397–18402. [PubMed: 16344477]
- (31). Steinbach PJ. Inferring Lifetime Distributions from Kinetics by Maximizing Entropy using a Bootstrapped Model. *J. Chem. Inf. Comp. Sci.* 2002; 42:1476–1478.
- (32). Steinbach PJ, Ionescu R, Matthews CR. Analysis of Kinetics Using a Hybrid Maximum-Entropy/Nonlinear-Least-Squares Method: Application to Protein Folding. *Biophys. J.* 2002; 82:2244–2255. [PubMed: 11916879]
- (33). Russell BS, Bren KL. Denaturant Dependence of Equilibrium Unfolding Intermediates and Denatured State Structure of Horse Ferricytochrome *c*. *J. Biol. Inorg. Chem.* 2002; 7:909–916. [PubMed: 12203029]
- (34). Vecer J, Herman P. Maximum Entropy Analysis of Analytically Simulated Complex Fluorescence Decays. *J. Fluoresc.* 2011; 21:873–881. [PubMed: 20066479]
- (35). Rhoades E, Cohen M, Schuler B, Haran G. Two-state Folding Observed in Individual Protein Molecules. *J. Am. Chem. Soc.* 2004; 126:14686–14687. [PubMed: 15535670]
- (36). Chung HS, Louis JM, Eaton WA. Distinguishing between Protein Dynamics and Dye Photophysics in Single-molecule FRET Experiments. *Biophys. J.* 2010; 98:696–706. [PubMed: 20159166]
- (37). Chang IJ, Lee JC, Winkler JR, Gray HB. The Protein-folding Speed Limit: Intrachain Diffusion Times Set by Electron-transfer Rates in Denatured Ru(NH₃)₅(His-33)-Zn-cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:3838–3840. [PubMed: 12646702]
- (38). Wittung-Stafshede P, Lee JC, Winkler JR, Gray HB. Cytochrome *b*-562 Folding Triggered by Electron Transfer: Approaching the Speed Limit for Formation of a Four-Helix-Bundle Protein. *Proc. Natl. Acad. Sci. U.S.A.* 1999; 96:6587–6590. [PubMed: 10359755]
- (39). Lee JC, Chang IJ, Gray HB, Winkler JR. The Cytochrome *c* Folding Landscape Revealed by Electron-transfer Kinetics. *J. Mol. Biol.* 2002; 320:159–164. [PubMed: 12079375]
- (40). Yamada S, Ford NDB, Keller GE, Ford WC, Gray HB, Winkler JR. Snapshots of a Protein Folding Intermediate. *Proc. Natl. Acad. Sci. U.S.A.* 2013; 110:1606–1610. [PubMed: 23319660]
- (41). Borgia A, Williams PM, Clarke J. Single-Molecule Studies of Protein Folding. *Ann. Rev. Biochem.* 2008; 77:101–125. [PubMed: 18412537]
- (42). Kuzmenkina EV, Heyes CD, Nienhaus GU. Single-Molecule Förster Resonance Energy Transfer Study of Protein Dynamics Under Denaturing Conditions. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:15471–15476. [PubMed: 16221762]
- (43). 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. U.S.A.* 2000; 97:5179–5184. [PubMed: 10792044]
- (44). Laurence TA, Kong XX, Jager M, Weiss S. Probing Structural Heterogeneities and Fluctuations of Nucleic Acids and Denatured Proteins. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:17348–17353. [PubMed: 16287971]
- (45). Kuzmenkina EV, Heyes CD, Nienhaus GU. Single-Molecule FRET Study of Denaturant Induced Unfolding of RNase H. *J. Mol. Biol.* 2006; 357:313–324. [PubMed: 16426636]
- (46). Merchant KA, Best RB, Louis JM, Gopich IV, Eaton WA. Characterizing the Unfolded States of Proteins Using Single-Molecule FRET Spectroscopy and Molecular Simulations. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:1528–1533. [PubMed: 17251351]

- (47). Huang F, Sato S, Sharpe TD, Ying LM, Fersht AR. Distinguishing Between Cooperative and Unimodal Downhill Protein Folding. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:123–127. [PubMed: 17200301]
- (48). Tezuka-Kawakami T, Gell C, Brockwell DJ, Radford SE, Smith DA. Urea-induced Unfolding of the Immunity Protein Im9 Monitored by spFRET. *Biophys. J.* 2006; 91:L42–L44. [PubMed: 16798813]
- (49). Schuler B, Lipman EA, Eaton WA. Probing the Free-energy Surface for Protein Folding with Single-Molecule Fluorescence Spectroscopy. *Nature.* 2002; 419:743–747. [PubMed: 12384704]
- (50). 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. [PubMed: 18507381]
- (51). Pugh SD, Gell C, Smith DA, Radford SE, Brockwell DJ. Single-Molecule Studies of the Im7 Folding Landscape. *J. Mol. Biol.* 398:132–145. [PubMed: 20211187]
- (52). Kinoshita M, Kamagata K, Maeda A, Goto Y, Komatsuzaki T, Takahashi S. Development of a Technique for the Investigation of Folding Dynamics of Single Proteins for Extended Time Periods. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:10453–10458. [PubMed: 17563378]
- (53). Choi J, Kim S, Tachikawa T, Fujitsuka M, Majima T. Unfolding Dynamics of Cytochrome *c* Revealed by Single-Molecule and Ensemble-Averaged Spectroscopy. *Phys. Chem. Chem. Phys.* 2011; 13:5651–5658. [PubMed: 21305089]
- (54). Braun M, Rubio IG, Thony-Meyer L. A Heme Tag for *in vivo* Synthesis of Artificial Cytochromes. *Appl. Microbiol. Biotechnol.* 2005; 67:234–239. [PubMed: 15834717]
- (55). Asher WB, Bren KL. Use of a Heme Fusion Tag for Protein Purification and Quantification. *Protein Sci.* 2010:1830–1839. [PubMed: 20665691]

Figure 1.

(a) Scheme for immobilization of K99C-AF488 to a PEG-coated quartz coverslip. The arrow between the AF488 and the heme illustrates how the proximity changes between the two chromophores as the protein unfolds. (b) Images of K99C-AF488 in the presence of various concentrations of denaturant. Data was collected using a 100 μ s integration time per pixel. The horizontal dimension is approximately 10 μ m.

Figure 2.

Representative fluorescence intensity and lifetime time trace for an individual molecule of K99C-A488 at 2.0 M GuHCl. (a) 50-ms binned intensity data (gray) with the intensity reconstructed by the change point algorithm (red) showing six different intensity states ($I_1 - I_6$). (b) TCSPC histograms (red markers) and the MLE fits (black line) for extracting the lifetimes for the five dye fluorescence intensity states ($I_2 - I_6$) from (a), compared with the background intensity (I_1). Photon counts for each lifetime state are depicted below the lifetime values. (c) Extracted fluorescence lifetime (blue) as a function of time overlaid onto the 50-ms binned intensity trace (gray).

Figure 3.

(a) Distribution of SM fluorescence lifetime states of K99C-AF488 at various concentrations of GuHCl. (b) Ensemble TCSPC histograms of 150 nM K99C-AF488 in solution (red), compared with TCSPC histograms of photons collected from SMs (black). The SM photon histogram in (b) includes only the photons used to generate the histograms in (a). (c) MEMexp fits of the ensemble decay curves (red line in (b)).

Figure 4.

Two-dimensional histograms of transitions between lifetime states in SM time traces of K99C-AF488 at 1.75, 2.00, 2.25 and 2.50 M GuHCl. The data are plotted in 0.2 ns bins for ease in presentation. Points appearing on the diagonal correspond to small (but non-zero) changes in lifetime that both fall within a given bin.