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Fluorescence Correlation Spectroscopy of Fast Chain Dynamics within Denatured Protein L

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Abstract

Intramolecular dynamics in the denatured state of a protein are of importance for protein folding. Native-like contact formation within the ensemble of denatured conformations of a protein may guide its transformation towards the native conformation. The efficiency of folding is thus dependent on the diffusion of chain fragments, which facilitates contact formation. Herein we investigate intramolecular diffusion of denatured molecules of the small two-state-folding protein L with fluorescence correlation spectroscopy (FCS). We utilize the specific quenching of the fluorescence of the oxazine dye Atto655 labeling a cysteine at position 64 (the C-terminus of the protein) by the side chain of a tryptophan at position 47. FCS measurements allow us to probe processes ranging in time-scales from tens of nanoseconds to seconds. Two fast photo-physical processes can be distinguished in the fluorescence correlation curves. The slower of the two is found to be due to triplet dynamics, while the faster process is attributed to the quenching of the Atto655 by the tryptophan upon transient ground-state complex formation. We study the dependence of the intrachain dynamics of the denatured protein on the concentration of the denaturant guanidinium chloride (GdmCl), and extract complex association and dissociation rates. While the dissociation rate does not depend on the denaturant, the association rate decreases as denaturant concentration is increased from 3 to 7 M GdmCl. This decrease in contact formation rate tracks the expansion of denatured protein L, measured in our previous work. Thus, the intramolecular diffusion coefficient calculated from the results is found to be essentially independent of the denaturant concentration over this range, even as the protein expands by more than 20 %.

Keywords

diffusion; fluorescence; fluorescence correlation spectroscopy; photoinduced electron transfer; protein folding

1. Introduction

Protein folding involves an intramolecular search for the correct configuration of the chain. The dynamics of chain reconfiguration may thus play a critical role in determining the rate of folding. Indeed, native-like contact formation within the ensemble of denatured conformations of a protein guides the transformation from unfolded conformations to the native one. Long range interactions are required for the formation of secondary structure

elements such as parallel beta strands. Such contacts are also essential for the formation of stable helical fragments and anti-parallel beta-sheets, which tend to be unstable in isolation.^[1]

It is therefore natural that protein-chain reconfiguration times and the related intramolecular diffusion coefficients have been under scrutiny for many years. Haas et al. pioneered time-resolved FRET measurements as means to study end-to-end dynamics of peptides.^[2] Their method, which detected changes in FRET efficiency during the lifetime of the fluorescent probes, was limited to peptides of less than 10 residues. Kiefhaber and coworkers^[3,4] and Eaton and coworkers^[5,6] introduced methods based on triplet photophysics, which allowed them to measure longer peptides. Both groups found that for peptides longer than 10 residues the end-to-end contact formation times scaled with length as expected for a random coil.^[7] Lapidus et al. showed that the rate of tryptophan triplet quenching by a cysteine depends both on diffusion and on an intrinsic reaction time, and by disentangling the two effects they were able to calculate an effective diffusion coefficient for chain ends of the order of $10^{-6} \text{ cm}^2 \text{ sec}^{-1}$.^[6] Contact formation measurements were more recently adapted to study dynamics within denatured single-domain proteins.^[8,9] Nettels et al.^[10] showed that intrachain dynamics within a protein can also be measured using a fluorescence correlation spectroscopy (FCS) technique based on FRET, which extends the original method of Haas et al.^[2] to time scales longer than the fluorescence lifetime.

FCS was also used to study intramolecular dynamics by Sauer and coworkers, who employed the transient quenching of oxazine dyes by a tryptophan residue as a probe. It was found that the quenching process is mainly static, involving the formation of a short-lived ground-state complex between a dye molecule and the side chain of a tryptophan residue,^[11] and the process was therefore suitable to study fast intramolecular contact formation of short peptides,^[12] as well as folding dynamics in a small protein.^[13]

Herein we employ FCS in conjunction with transient oxazine dye quenching to study the denatured-state dynamics of the small, 64-amino-acid protein L.^[14] We previously performed equilibrium single-molecule FRET experiments on this protein.^[15] FRET efficiency values of thousands of individual double-labeled protein L molecules were measured. These values were used in order to calculate the radius of gyration of denatured protein L as a function of denaturant concentration, and a gradual expansion of the protein was observed. A similar expansion was also evident from values of the hydrodynamic radius of the protein extracted from FCS measurements. This expansion was interpreted using the theory of globule-coil transition in polymers. Our results were later confirmed by Merchant et al.,^[16] and similar observations were made on many other proteins (for reviews see [17, 18]). Our current measurements suggest that rates of contact formation between the dye Atto655 and a tryptophan residue show an inverse trend to the denatured-state expansion, indicating that the intramolecular diffusion coefficient is independent of denaturant concentration between 3 and 7 M guanidinium chloride (GdmCl).

2. Results

2.1. Bulk Fluorescence Measurements

We prepared molecules of protein L with the mutation G64C, and labeled them with the dye Atto655 maleimide. Our version of protein L contains a tryptophan residue at position 47 (Trp47), and we expected this residue to quench the fluorescence of the dye, making it a reporter on loop closure within the polypeptide chain in protein L. Indeed, observation of the structure of the labeled protein (Figure 1a) indicated the possibility of contact between the Atto655 dye labeling residue 64 with the partially solvent-exposed side-chain of Trp47. Further, in steady-state fluorescence measurements of the labeled protein as a function of

denaturant concentration it was found that the fluorescence intensity of the dye increased dramatically as the protein unfolded (Figure 1 b). Taken together, these two observations allowed us to conclude that Atto655 quenching reports on contact formation, which is more likely to occur in the folded state, and becomes less probable as the protein denatures.

Time-correlated single-photon counting measurements of the emission of the free dye and the labeled protein indicated exponential fluorescence decay (Figure 2). The lifetime of the free dye in buffer was found to be ~1.9 nsec, in good agreement with previously published results.^[11] The lifetime of the dye on the labeled protein was larger than that of the free dye, both in buffer (where quenching is maximal) and in 6 M GdmCl. Importantly, no fast components appeared on the decay curve, indicating that within the limits of the time resolution of our instrument (whose instrument response function width is 300 ps) quenching of the Atto655 emission does not seem to have a significant dynamic component. Thus, the strong fluorescence quenching of the dye on protein L must be attributed to static quenching by tryptophan, as already shown in the literature.^[11, 12] This static quenching is partially alleviated as the concentration of GdmCl is increased, leading to enhanced emission from the labeled protein. However, as will be shown below, contact-formation induced quenching of Atto655 by tryptophan occurs also in the fully denatured state of protein L.

2.2. FCS Measurements

Correlation curves resulting from FCS measurements of labeled G64C molecules, taken over a range of denaturant concentrations, are shown in Figure 3. Complex dynamics are evident in these curves at times shorter than the diffusion time (Figure 3 b). The experimental correlation functions were fitted according to the model described in the Experimental Section [Eq. (3)]. Two exponential processes were required in order to obtain a satisfactory fit. Figure 3 c shows correlation curves obtained from measurements of the free Atto655 dye in solution, and of the G64C W47F mutant, which lacks the tryptophan residue. The fitting of these correlation curves required only a single exponential process, since the faster of the two exponential processes was essentially absent. We thus confirmed that the fast process was caused by the selective quenching of the Atto655 dye by the side-chain of Trp47.

Experiments performed at different laser power levels can help recognizing processes mediated through the excited singlet state of the fluorescent dye, since increased laser power may lead to increased population of this state. Specifically, dynamics involving intersystem crossing from the excited singlet state to a triplet state, which are expected to be laser-power dependent, can be identified in such experiments. We therefore performed FCS experiments on the free dye and on labeled G64C molecules at a series of laser power levels. The correlation curves were fitted to Equation (3), and the parameters describing the two exponential processes were extracted. Figure 4 shows the dependence of these parameters on laser power. Measurements were done on G64C in 6 M GdmCl, and on the free Atto655 dye in either 0 M or 6 M GdmCl. We observed that the amplitude of the fast process is independent of power level (panel a). On the other hand, the amplitude of the slow process grows monotonically with excitation intensity, saturating at high enough power levels (panel c). This amplitude could be fitted to the equation: $K_{\text{slow}} = \frac{CI}{1+I/I_s}$, where I_s is the saturation power and C is a constant (red line in panel c; $R^2=0.981$). The fit suggests that optical saturation is the likely cause of the shape of the curve, with an I_s value of ~ 550 μW . A very similar behavior was found in FCS curves of free Atto655 in 6 M GdmCl, but not in buffer, where the dependence on power was much weaker. As for the lifetimes of the two processes, while the lifetime of the slow process changes *nonlinearly* with the power levels (Figure 4d), that of the fast process is insensitive to the excitation power (Figure 4 b).

We next conducted FCS measurements of the protein L over a range of GdmCl concentrations. These experiments were performed at a laser power of 120 μ W. We focus here on the fast process as the parameters of the slow process were fully accounted by the free dye (Figure 4) and did not change within the measured GdmCl concentration (data not shown). Figure 5 shows some values of the amplitude of the fast process, which was found to decrease with GdmCl concentration (panel a), and of the lifetime, which seemed to be independent of GdmCl concentration (panel b).

3. Discussion

3.1. Interpretation of the FCS Curves

Three dynamic processes can be clearly distinguished in the FCS curves: First, a slow, ~ 0.5 –1 msec long process due to diffusion of labeled protein molecules through the laser beam; second, an exponentially decaying process with a lifetime of ~ 8 –16 μ sec, which we will refer to as the “slow” process; and third, another exponentially decaying process with a faster lifetime of 0.2–0.5 μ sec, hereby referred to as the “fast” process. The temporal separation of the three processes by 1–2 decades allows us to clearly distinguish them. Moreover, the short lifetimes of the two exponential processes makes their determination from the FCS curve relatively insensitive to parameters of the experimental system, such as refractive index.^[19]

In order to further analyze the FCS curves, we first identify the photophysical processes that give rise to the slow and fast dynamic processes. The amplitude of the slow process grows nonlinearly with laser intensity, both in measurements of the denatured labeled protein and of the free dye in denaturant solution (Figure 4 c). Thus, we identify this process as involving triplet state dynamics of the Atto655 dye. Ruttinger^[20] showed that optical saturation of the Atto655 dye occurs at an excitation power of ~ 100 μ W, which is smaller than the ~ 550 μ W saturation power found here. However, a pulsed laser source was used by Ruttinger, as opposed to the continuous-wave laser used here, which might explain the difference in saturation powers. As already noted, the fast process is absent in a protein that lacks the tryptophan residue and in the free dye (Figure 3 c). The amplitude of the fast process and its lifetime are insensitive to laser power (Figure 4 a,b), but sensitive to GdmCl concentration (Figure 5 a,b). Thus we can conclude that the fast process originates from quenching of the Atto655 dye by Trp47, which depends on the conformation of the protein.

Once we identified the two processes, we can devise a model that will allow us to obtain the rate of contact formation between the dye and Trp47. Following Neuweiler et al.,^[12] we make the following assumptions: a) the dye–tryptophan complex is non-fluorescent, due to a photoinduced electron transfer process, which is much faster than any of the dynamic processes that appear in the FCS curves; b) Complex formation does not influence the diffusion time of protein molecules; c) Reorientational motion of the protein or dye does not play a role in the measurements.

Widengren et al.^[21] showed that the dynamics of a system similar to the one discussed here can be described by a simple reduced kinetic scheme, which is shown in Figure 6. The scheme involves only three states, namely the singlet (S), triplet (T) and dye–tryptophan complex (C) states. Ground and excited singlet states are not explicitly treated in this scheme. Rather, by invoking fast equilibrium between the ground and excited singlet states, effective rate constants are derived, which interconnect the three above-mentioned states. Since the fast and slow process differ by at least an order of magnitude, we can make the further assumption that the rates related to the reaction $S \leftrightarrow C$ are much larger than rates related to the reaction $S \leftrightarrow T$. We can then write approximately [Eq. (1)]:

$$\frac{1}{\tau_{\text{fast}}} \approx k_{\text{ass}} + k_{\text{diss}} \\ K_{\text{fast}} = \frac{C}{1-C-T} \approx k_{\text{ass}}/k_{\text{diss}} \quad (1)$$

where k_{ass} and k_{diss} are the complex association and dissociation rates, respectively, which can be readily calculated from Equation (1), and are shown in Figure 7 as a function of GdmCl concentration. The resultant association rates are significantly slower than the dissociation rates—by an order of magnitude. While the dissociation rates seem independent of the GdmCl concentration, the association rates decrease monotonically with this concentration. We find that the association rates of the denatured protein L chain vary between ~ 0.6 and $\sim 0.3 \mu\text{sec}^{-1}$.

It is important to mention here that the folded-state dynamics do not contribute to the numbers extracted above. As can be deduced from Figure 1 b, the number of photons emitted by the protein in the folded state is ~ 5 times smaller than the number of photons emitted in the unfolded state. Now the contribution of each component in the FCS curve of a multi-component mixture gets multiplied by its relative number of photons *squared*.^[22] This means that even when half of the population is in the folded state, the contribution of this fraction to the FCS curve is 25 times smaller than the contribution of the unfolded fraction, and can be neglected.

3.2. Extracting Intrachain Diffusion Coefficients

The observed quenching rates in our measurements should in principle reflect both a diffusion-controlled association rate and a reaction-limited rate which depends on the actual quenching mechanisms.^[6] However, it has been shown that the quenching mechanism in oxazine dyes involves photoinduced electron transfer within a ground-state complex, which is much faster than the diffusion-limited rate for complex formation.^[12] This allows us to interpret the observed quenching rates as the contact formation rates with good accuracy. It should be noted that Doose et al.^[23] calculated that on the average 3 ± 1.5 encounters between the dye MR121 and tryptophan are required for the formation of a complex. This estimate is based on comparison of the quenching rate obtained from an FCS measurement with a calculated diffusion-limited collision rate based on the well-known Smoluchowski expression. In principle, this estimate implies that our reported values for the association rates should be multiplied by a factor of ~ 3 . However, due to the large reported uncertainty in this estimate, as well as additional potential uncertainties in the estimation of the diffusion-limited rate, we elect not to correct our association rates. In any case, such a correction should not affect the results reported below qualitatively.

Since an increase of the GdmCl concentration expands the protein chain^[15] and significantly alters the viscosity (by a factor of about two within the measured concentration range), one should expect the association rate to decrease as GdmCl concentration increases. On the other hand, dissociation rates of the quenched complex should, in principle, show little dependence on either viscosity, or the overall size of the protein chain. Our results for the dependence of the dissociation and association rates on GdmCl (Figure 7) are in agreement with these expectations. Doose et al.^[23] found a marked effect of GdmCl on the dissociation rates of complexes of the dye MR121 with tryptophan. The lack of such an effect in our study might stem from a difference between MR121 (which is known to be similar to Atto655) and the Atto655, or might be due to the protein environment, which modifies the interaction of GdmCl with the complex.

The mean first-passage time theory of Szabo, Schulten and Schulten^[7] provides an expression for the association rate of the chain ends of a polymer by treating their motion as

a diffusion process on a 1D potential. For a Gaussian chain, this expression reads as follows [Eq. (2)].^[6]

$$\frac{1}{k_{\text{ass}}} = \frac{3\langle r^2 \rangle^{3/2}}{(6/\pi)^{1/2} D \alpha} \quad (2)$$

where the mean-squared distance between probes, $\langle r^2 \rangle$, is proportional to the number of residues separating the probe, D is the relative diffusion coefficient of the chain ends, and α is the distance between probes at which reaction occurs. Contact formation dynamics should be affected by chain flexibility as well as by solution viscosity, which will both change D .

In our previous work on protein L^[15] we used single-molecule FRET histograms to extract the mean-squared end-to-end distance of denatured protein molecules, $\langle R_{ee}^2 \rangle$, as a function of the GdmCl concentration. A strong dependence of $\langle R_{ee}^2 \rangle$ on denaturant concentration was found, as shown in the inset to Figure 8. The measured $\langle R_{ee}^2 \rangle$ values can be readily used to calculate the mean-squared end-to-end distance of the 17-amino-acid internal loop, using the relation: $\langle r^2 \rangle = (17/64) \langle R_{ee}^2 \rangle$. This calculation ignores the contribution of the linker of the Atto655 dye. Unfortunately, the structure of this dye is proprietary, and we cannot make a correction for linker length. As for α , we will adopt the value of ~0.6 nm, which was deduced for complex formation of the oxazine dye MR121 with tryptophan, based on molecular dynamics simulations.^[24] Using Equation (2), we can now calculate the intrachain diffusion coefficient D . Figure 8 shows D as a function of denaturant concentration. Surprisingly, we find that D of denatured protein L is essentially insensitive to the denaturation conditions, taking an average value of $4.2 \pm 0.4 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$.

Intrachain diffusion-coefficient values of the same order of magnitude have been reported for protein L above 3 M GdmCl by Lapidus and coworkers, who used a different technique, namely, the quenching of the triplet state of tryptophan by a cysteine residue.^[9, 25] For comparison, we show these values as well in Figure 8 (black symbols). The trend with GdmCl concentration is not as easy to discern with this data as with our data. Further, these authors extracted their diffusion coefficient values based on simulated end-to-end distances. In the current work we used values which we directly obtained from our experimental measurements, and which differ significantly from the simulated values. Nevertheless, it is reassuring that two different experiments provide diffusion coefficient values that agree within a factor of two. These values are quite a bit smaller than values of D calculated from measurements on peptides.^[6] Interestingly, larger intrachain diffusion coefficients than found for protein L were also obtained by Schuler and coworkers in their measurements of denatured cold shock protein (Csp) molecules,^[10] although contact formation rates measured on the same protein by Buscaglia et al.^[8] seem to be more in line with the numbers reported here. Taken together, all these findings suggest that values of intrachain diffusion within proteins may reflect the specific amino-acid sequence of the probed loop, and also the specific context, that is, the structure taken by the rest of the chain, which might not be completely random even at high denaturant concentrations.

In fact, the intrachain diffusion coefficient should depend both on the internal friction generated by the probed loop itself and its interaction with other parts of the chain, and on the solution viscosity. It has been suggested that D should be proportional to the inverse of the sum of the contributions of internal friction and external friction: $D \propto 1/(f_{\text{int}} + f_{\text{ext}})$.^[2, 26] The external friction takes the familiar Stokes form: $f_{\text{ext}} = 6\pi\eta R$, where η is the solvent viscosity and R is the radius of the diffusing object, but it is not so clear what form the

internal friction should take.^[26] Nettels et al. corrected for the effect of external friction by simply multiplying values of D by the solvent viscosity,^[10] but this type of correction ignores the additive nature of the internal and external frictions. In fact, if one corrects back the D values of Nettels et al., one finds that they also vary very little above 3 M GdmCl, in a similar fashion to the trend found here.

4. Conclusions

Herein we reported FCS measurements of conformational dynamics within the relatively small protein L, using the fast quenching of the Atto655 by tryptophan as our tool. Two fast photophysical processes could be distinguished in the fluorescence correlation curves. We were able to verify that the slower of these is related to triplet-state population and depopulation of the Atto655 dye in GdmCl solutions (where the photophysics is markedly different than in buffer). The faster and more interesting process was attributed to the quenching of the Atto655 by Trp47 upon contact formation. We studied the dependence of the intrachain dynamics of the denatured protein on denaturant concentration. Contact formation between distant sites on a chain may be a rate-limiting step for proteins to fold and it was found here to be rather slow and to occur on a timescale of several microseconds. Using the Szabo, Schulten and Schulten theory and the dimensions of the protein derived from single-molecule FRET experiments, we could estimate the intrachain diffusion of the denatured state of the protein over a range of denaturing conditions. The diffusion coefficient of the chain is surprisingly insensitive to the denaturant concentrations over the range studied. We conclude that above 3 M GdmCl the dynamics of denatured protein L remain essentially constant, even in the face of a large change in both solvent viscosity and overall dimensions. The fact that D values don't change over a range of GdmCl concentrations in which the solvent viscosity increases by almost a factor of two may in principle be explained by changes in in-trachain diffusion which accidentally cancel the viscosity effect. However, we tend instead to invoke Occam's razor, and accept the simpler explanation that *intramolecular diffusion is in fact dominated by an essentially constant internal friction*. It will be interesting to find out what the microscopic origins of this internal friction are.

Experimental Section

Chemicals

Ultrapure guanidinium chloride (8 M GdmCl) was purchased from Pierce. The viscosity of GdmCl solutions was determined through measurements of their index of refraction.^[27] Ultra-pure phosphate acid and base reagents were purchased from Fluka.

Protein Samples and Labeling

A peT-7 plasmid containing a histidine-tagged version of protein L was a kind gift of David Baker (University of Washington, Seattle). This version of protein L contains a tryptophan residue at position 47, and has the following amino-acid sequence:

HHHHHHAMEEVTIKANLIFANGSTQTAEFKGT-

FEKATSEAYAYADTLKKDNGEWTVDVADKGYTLNIFAG. Two mutants of the protein were used in this experiment, G64C, in which a glycine residue at position 64 was replaced by cysteine (using the PCR primer (5') CTT TAA ATA TTA AAT TTG CTT GCT AGA TGC ATG GAG GAA ACG CGT AAG GAT C), and G64C, W47F, in which in addition the tryptophan residue was mutated to phenylalanine (using the PCR primer (5') AAG AAA GAC AAT GGA GAA TTT ACT GTC GAC GTT GCA GAT AAA GG). For generation of the desired mutant proteins, the plasmid with the point mutations was introduced into competent BL-21 (Pliz) bacteria cells. The bacteria cells were grown in

Luria broth (LB) culture with ampicillin (1 mM) and chloramphenicol (1 mM) antibiotics. Over-expression was induced by IPTG (1 mM), and was allowed to continue for three hours at room temperature. After this time, cultures were centrifuged (4000 rpm), sonicated and purified using HiTrap Ni-column (Amersham Pharmacia). Elution of the histidine-tagged proteins from the nickel column occurred at imidazole concentrations ranging between 80 mM and 300 mM, depending on the specific mutant. Proteins were stored for further use at -80°C in 10 % glycerol, as well as EDTA (200 μM) and DTT (20 μM).

Protein molecules were labeled using a single fluorescent probe (Atto655 maleimide, Attotec) that was attached to the inserted cysteine residue. Labeling was performed in phosphate buffer under oxygen-free and reducing conditions [using tris(2-carboxy-ethyl)phosphine]. The labeled proteins were purified using two PD-10 size exclusion columns (Amersham Pharmacia). Further purification steps included dialysis (Slide-A-Lyzer, cutoff of 3500 Da, Pierce) and an anion-exchange column (MonoQ HR 5/5 column, Amersham Pharmacia). Labeled protein samples were kept and measured in phosphate buffer (50 mM, pH 7.4). The steps of over-expression, purification and labeling were routinely verified using SDS gels (15 %) and mass spectrometry.

Fluorescence Spectroscopy and Lifetime Measurements

The fluorescence emission spectra of labeled proteins, as well as that of the free dye, were collected using a Fluorolog spectrofluorometer (Jobin-Yvon) at a series of GdmCl concentrations. Time-resolved fluorescence measurements of these samples were performed using a FluoroCube time-correlated single photon counting instrument (Jobin-Yvon). The labeled proteins were excited using a 635 nm LED. Emission was detected at 680 nm.

Fluorescence Correlation Spectroscopy (FCS)

FCS experiments were performed using a home-built confocal microscope equipped with a water-immersion UplanApo 60x NA 1.2 (Olympus) objective. The collar setting of the objective was set to 0.17 throughout all measurements. The sample was illuminated by a linearly polarized HeNe ion laser (model 25LHP073, Melles Griot), focused through the objective. Fluorescence collected through the objective was passed through a dichroic mirror (Z633RDC, Chroma) and two long-pass emission filters (Z7889 OSDC from Oriel and RG645 from Schott). The fluorescence was then focused by the microscope tube lens onto a 50 μm pinhole. The fluorescence signal was then split onto two photon-counting avalanche photodiode (Perkin-Elmer Photoelectronics) using a 50/50 non-polarizing beam-splitter (Unice E-O Services). Cross-correlation curves were generated and registered using a hardware correlator (Flex02-12D, Correlator.com). Samples were typically measured at a protein concentration of 10 nM within sealed cells constructed from two cover slides.

Fitting of Correlation Curves

The experimental correlation functions of protein L molecules in various solutions were fitted to a model that included diffusion of molecules through a three-dimensional Gaussian illumination volume and two intramolecular processes showing exponential decay [Eq. (3)]:^[22, 28]

$$G(t) = \frac{1}{N} \left[1 + \frac{t}{\tau_d} \right]^{-1} \left[1 + \frac{t}{\omega^2 \tau_d} \right]^{-1/2} [1 + K_{\text{fast}} \exp(-t/\tau_{\text{fast}}) + K_{\text{slow}} \exp(-t/\tau_{\text{slow}})] \quad (3)$$

where K_{slow} and K_{fast} are the pre-exponential factors (or amplitudes) related to the fast and slow processes and τ_{slow} and τ_{fast} are their lifetimes, respectively. N is the total average

number of molecules in the sample volume. τ_d is the mean diffusion time of a molecule through this volume, and ω is the aspect ratio of its axial and radial dimensions. The correlation curve of free Atto655 in buffer at a low power level (20 μ W) showed only a single process indicative of diffusion of the dye molecules through the focused laser beam, with no further exponential processes (data not shown).

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Figure 1.

Quenching of Atto655 by the Trp47 in protein L. a) Schematic of the structure of the G64C mutant of protein L labeled with Atto655, based on protein data bank file 2PTL. Trp47, which is partially exposed, is colored in purple. The possibility of contact formation between this residue and the Atto655 dye attached to residue 64 is evident. b) GdmCl dependence of the emission of Atto-655 labeled protein L molecules. Atto655 emission of the G64C construct (red squares) is efficiently quenched in the native state of the protein, but seems to increase in a cooperative fashion upon unfolding. Emission of the free Atto655 dye (not shown) is essentially unaffected by GdmCl concentration. The continuous line is a two-state fit to the experimental points.

Figure 2. Time-correlated single-photon counting measurements of the free Atto655 dye in buffer and of G64C labeled with Atto655 at two GdmCl concentrations, 0 M and 6 M. The instrument response function is also shown. It is evident that no fast components due to dynamic quenching appear in the fluorescence decay curve of the labeled protein.

Figure 3.

FCS of labeled protein molecules. a) Correlation curves of labeled G64C molecules at a range of GdmCl concentrations. b) The curves measured at 3 M GdmCl (green) and 7 M GdmCl (red) are plotted over a shorter timescale to stress the fast dynamical processes. Dots are data points, while continuous lines are fits. The lower panels show the residuals of the fits in matching colors. c) Fluorescence correlation curves of the labeled G64C W47F mutant (green) and of the free Atto655 dye (red). In this panel the figures show fits of the correlation curves to a model which includes a diffusion process and a single exponential process. In contrast to curves in panel b, the fast exponential process, on the order of hundreds of nano-seconds, is essentially absent from these curves, due to the absence of the quenching tryptophan residue. The lower panel shows the residuals of the fits in matching colors. All data in this Figure were taken with a laser power of 120 μ W.

Figure 4.

Parameters of the exponential dynamic processes in FCS curves of G64C and of the free dye as a function of laser power. a) amplitude of the fast process (K_{fast}) in protein FCS curves. b) Apparent lifetime of the fast process. c) Amplitude of the slow process (K_{slow}) in protein FCS curves (green squares) and in curves measured on free dye in buffer (yellow circles) as well as in 6 M GdmCl (red squares). d) Apparent lifetime of the slow process in labeled protein FCS curves (green squares) and of the free Atto655 dye in buffer (yellow circles) and in 6 M GdmCl (red squares). In panel (c), the red line is a fit of the slow process amplitude values to an equation describing optical saturation, as discussed in the text. Error bars shown are calculated from three independent experiments.

Figure 5. Parameters of the fast dynamic process in FCS curves of G64C as a function of the GdmCl concentration. a) Amplitude of the fast process. b) Apparent lifetime of the fast process. The error bars were calculated from three independent experiments.

Figure 6.

Proposed simplified diagram for the Atto655 quenching via complex formation upon contact with a tryptophan residue, based on the analysis of ref. [21]. S and T are effective singlet and triplet states, respectively, and C is the dye–tryptophan complex. k_{ISC} and k_{31} are the intersystem crossing and triplet relaxation rates, respectively, while k_{ass} and k_{diss} are the association and dissociation rates of the Atto655–tryptophan complex.

Figure 7. Dependence of dye-tryptophan complex dissociation (a) and association (b) rates on the GdmCl concentration. The rates were calculated as described in the text.

Figure 8. Intrachain diffusion coefficient of denatured protein L as a function of the denaturant concentration. The diffusion-coefficient values (gray squares) were calculated from the association rates using Equation (2), and values of the mean-squared end-to-end distance based on single-molecule FRET measurements from ref. [15] (shown in the inset). For comparison, we also show the diffusion-coefficient values reported by Waldauer et al.^[25] (black circles).