Quantitative Fluorescence Correlation Spectroscopy Reveals a 1000-Fold Increase in Lifetime of Protein Functionality

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ABSTRACT We have investigated dilute protein solutions with fluorescence correlation spectroscopy (FCS) and have observed that a rapid loss of proteins occurs from solution. It is commonly assumed that such a loss is the result of protein adsorption to interfaces. A protocol was developed in which this mode of protein loss can be prevented. However, FCS on fluorescent protein (enhanced green fluorescent protein, mCherry, and mStrawberry) solutions enclosed by adsorption-protected interfaces still reveals a decrease of the fluorescent protein concentration, while the diffusion time is stable over long periods of time. We interpret this decay as a loss of protein functionality, probably caused by denaturation of the fluorescent proteins. We show that the typical lifetime of protein functionality in highly dilute, approximately single molecule per femtoliter solutions can be extended more than 1000-fold (typically from a few hours to >40 days) by adding compounds with surfactant behavior. No direct interactions between the surfactant and the fluorescent proteins were observed from the diffusion time measured by FCS. A critical surfactant concentration of more than 23 μM was required to achieve the desired protein stabilization for Triton X-100. The surfactant does not interfere with DNA-protein binding, because similar observations were made using DNA-cutting restriction enzymes. We associate the occurrence of denaturation of proteins with the activity of water at the water-protein interface, which was recently proposed in terms of the "water attack model". Our observations suggest that soluble biomolecules can extend an influence over much larger distances than suggested by their actual volume.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) has developed (1–4) into a powerful method to analyze real-time diffusional properties of molecules, molecular binding and affinity, and molecular dynamic processes. The fluorescence amplitude fluctuations can be directly acquired and the subsequent conversion to an autocorrelation trace (or cross correlation) is easily performed. A large number of dynamic models have been theoretically analyzed and experimentally verified (5–9) and highlight the strength of FCS. Molecular diffusion is just one of the dynamic processes that can be successfully studied with this technique, and theoretical models have been proposed to describe molecular diffusion in different regimes (1,2,10). A model for free, three-dimensional (3D) diffusion is well established (10). The translational diffusion time and the average number of molecules in the measurement volume can be directly obtained from FCS measurements. It turned out, however, that reproducible measurements of the concentration of proteins in dilute, single molecule concentrations are not easy to achieve, due to a gradual loss of protein and/or denaturation of the protein. The average residence time in a measurement volume can be accurately and reproducibly determined.

The inaccuracy in the number determination of proteins at dilute, nanomolar concentrations is commonly interpreted as interfacial protein loss and significantly interferes with the ability to measure quantitative properties of, for instance, protein binding parameters and protein aggregation constants. This, in practical terms, limits the consistency of FCS results. We concluded that to correctly assess both concentration and residence times in dilute, nanomolar protein solutions under ambient condition (room temperature 19–23°C), special care should be taken to prevent such interfacial protein loss. We have used hydrophobic coverslips to prevent protein adsorption to surfaces and tested this approach down to the single molecule regime. It was noticed that despite the measures taken to prevent protein adsorption, a gradual loss of fluorescence occurred for dilute fluorescent protein solutions. Since no fluorescent molecules could be detected at the surfaces, even with single molecule imaging capability, we concluded that the proteins were losing their fluorescence functionality, possibly as a result of denaturation. Usually, the stability of proteins has been investigated at relatively high concentrations (e.g., from millimolar to micromolar) protein solutions, because common techniques, such as circular dichroism, calorimetry, and nuclear magnetic resonance, are not sufficiently sensitive for nanomolar protein solutions. At such high concentrations, however, proteins may tend to stabilize each other and a minor loss of functional protein may be unnoticed.

In this work, we used three mutant fluorescent proteins (FPs), one a red-shifted variant of wild-type green fluorescent protein (GFP) (11), known as enhanced GFP (eGFP) and two monomeric, red fluorescent derivatives of DsRed, known as mCherry and mStrawberry (12) to study cause and characteristics of protein loss in nanomolar solutions. FCS has been successfully applied before (13–15) to study the kinetic

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stability and biological function of fluorescent proteins in aqueous solution. The stability of FPs is directly related to their fluorescence output, which is in turn related to the integrity of the protein surrounding the fluorophore. We will demonstrate (vide infra) a gradual loss in the amplitude of the fluorescence emission in conjunction with an absence of interfacial protein loss and interpret this phenomenon as the result of a denaturation process. Subsequently we will show that the protein functionality lifetime in nanomolar protein solutions is increased >1000-fold in nonionic surfactant solution at room temperature without any other specific conditions, whereas the diffusion properties of the fluorescent proteins are unaltered by the presence of the surfactant. The latter observation suggests that the protein functionality lifetime is increased even without direct contact between protein and surfactant molecules. Subsequently, we show that a certain minimal surfactant concentration is necessary to be effective. To test whether this observation extends also to protein functionalities other than ''fluorescence'', we show that the presence of surfactant also stabilizes the function of restriction enzymes (related to the ''functionalities'' DNA recognition, binding, and cutting) over a period of 6 weeks.

MATERIALS

Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TFOS, product No. 448931), rhodamine 6G (R6G, product No. R-4127; molecular mass, 479 g/mole), and Triton X-100 (TX100, product No. T8787) were purchased from Sigma-Aldrich (St. Louis, MO). The other surfactant Nonidet P-40 (NP40, product No.11754599001) was purchased from Roche Applied Science (Indianapolis, IN). Purified bovine serum albumin (BSA, product No. B9001S) used to stabilize FPs, restriction enzymes *Fok*I (product No. R0109S, 4 units/μl), and *Ava*I (product No. R0152S, 10 units/μl) were purchased from New England Biolabs (Hitchin, UK). 5′ Alexa-488 55-mer oligonucleotide and its complementary strand were purchased from DNA Technology A/S (Risskov, Denmark). Milli-Q water was characterized as having a resistance of 18 MΩ and was further purified by filtration over a 0.2 μm filter. High-performance liquid chromatography (HPLC) grade water was purchased from Merck (Whitehouse Station, NJ) and used for all buffer preparations and dilution steps.

METHODS

Single molecule fluorescence confocal microscope

A single molecule fluorescence confocal microscope (smFCM) with imaging capability was redesigned to accommodate FCS measurements. The smFCM was described elsewhere (16,17). A brief description of the setup is presented in the on-line Supplementary Material (Data S1). An Ar^+/Kr^+ mixed gas laser was used for excitation of eGFP and Alexa488 (λ_{exc} = 488 nm) and mCherry and Strawberry (λ_{exc} = 568 nm.). The laser power was 20 μW in sample solution.

Coverslip surface passivation

The coverslips were first cleaned in 65% HNO_3, washed with milli-Q water, and then dried using N_2 gas. Protein adsorption at surfaces was prevented by passivating all coverslips with TFOS, which removes silanol groups at the coverslip surface (Supplementary Material, Data S1).

TFOS renders the surface highly hydrophobic, resulting in water contact angles above 120°, ensuring that 8 μl of aqueous sample solution, which was used for FCS measurements, forms a spherical droplet. TFOS-treated coverslips were washed with 100% ethanol and dried using N_2 gas before use.

On-line FCS analysis

A home-built computer program was written in LabVIEW (National Instruments, Austin, TX) to control the fluorescence microscope piezo nanopositioning stage and to record fluorescent amplitude time traces or spectra. The smFCM could be operated in four modes: i), fluorescent intensity imaging, ii), fluorescent spectral imaging, iii), intensity time trace for FCS, and iv), spectral time trace for sFCS (spectral FCS). Control software for FCS continuously recorded a fluorescence intensity time trace and calculated the autocorrelation trace for an adjustable preset time, which was 10 s. The autocorrelation trace was subsequently on-line fitted, with a nonlinear least-squares fitting procedure. Correlation analysis was coded in C++ language and integrated with the LabVIEW control software for fast calculations of the autocorrelation function or the cross-correlation function. Molecular residence times and average numbers of molecules in the focal volume could be rapidly determined by fitting each autocorrelation function to the generalized 3D diffusion model (10) in <2 s for a 10-s intensity time trace and a 128-point autocorrelation curve. Typical measurements consisted of 50 × 10-s time traces within 10 min. Temporal evolution of molecular residence times and average numbers of molecules in focal volume were directly monitored during measurements in a separate panel in the software display.

On-line FCS could directly be used to monitor molecular numbers and diffusion at different locations in the sample. Intensity time traces of surface-adsorbed proteins gave rise to high amplitude when started from a fresh position, which was followed by rapid intensity decay due to photobleaching of (immobilized) fluorescent molecules. In some experiments, the piezo scanner was used to start each FCS time trace at a fresh position, which was randomly distributed within an area of 50 × 50 μm^2 to take into account a nonuniformed surface coverage. All FCS measurements were repeated 50 times to acquire accurate statistics on all observations. Typically, sample drops were placed on a single TFOS-treated coverslip while the laser focus was moved from one drop to another. All other components of the smFCM were fixed, avoiding any variation in the excitation and observation volume (18).

All intensity time traces were saved to file and analyzed off-line at all available delay times, typically 8 × 10^5 time points. Concentrations of sample molecules were estimated from the background-corrected amplitudes of the FCS curves (8) and compared with the expected concentrations during sample preparation. Sample solutions were hermetically sealed in between two cover glasses using a silicone spacer to avoid evaporation. Volumes of measured solutions were always much smaller than the space in the sealed well so that droplets of sample solution were isolated from the silicone spacer material.

Buffers

PGI buffer was used to store FPs and contained 20 mM Na_3PO_4, (pH = 7.8), 300 mM NaCl, 10% glycerol and 60 mM imidazole (used for purification). Other buffers used were: a), 40 mM HEPES-NaOH buffer (pH 7.4), b), 25 mM Tris-HCl buffer (pH 7.4), c) phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 18.9 mM Na_2HPO_3, 1.8 mM KH_2PO_3, pH 7.5), and d), citric acid and potassium phosphate (CP) buffer (13–15) (10 mM

citric acid, 100 mM K_3PO_4, pH 7.0). TEK buffer, which was used to store restriction enzymes. consisted of 10 mM Tris, 0.1 mM EDTA and 50 mM KCl (pH 7.6). TEKT buffer was similar to TEK buffer with the addition of 0.23 mM TX100. NEBuffer 4 (New England Biolabs) was used for all DNA restriction reactions and consisted of 50 mM CH_3COOK, 20 mM Tris-acetate, 10 mM $(CH_3COO)_2Mg$, and 1 mM DL-dithiothreitol (pH = 7.9). HPLC grade water was used in all sample preparations.

Purification of fluorescent proteins

The eGFP gene was fused to N-terminal (His)9- and HA-tags and inserted into the pET17B vector (Novagen, San Diego, CA), removing the pET17B T7-tag. Plasmids pRSET-B-mCherry and pRSET-B-mStrawberry, containing (His)6-Xpress-tagged mCherry and mStrawberry, respectively, were kind gifts of Roger Y. Tsien (University of California, San Diego) (12). Recombinant proteins were produced by overexpression in *Escherichia coli* strain BL21(DE3) and subsequently purified using Ni-NTA chromatography (Qiagen, Valencia, CA) in PGI buffer. Protein concentrations were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Preparation of FP sample solutions

Preparation of fluorescent protein samples was as follows: 2 μl of stock solution of 2.5 μM eGFP in PGI buffer was first 10× diluted in 23 μM (i.e., 0.002% (v/v)) TX100 solution. Next, this stock solution was 25× diluted using HPLC grade water or other buffers, producing 10 nM eGFP solutions. Five 100 μl solutions (labeled FS1-5) 34 days old and of five freshly prepared solutions (labeled FFS1-5) were prepared containing 2.5 nM eGFP and different concentrations of TX100. TX100 concentrations in sample (F)FS1 to sample (F)FS5 were 2.3 μM, 23 μM, 230 μM, 2.3 mM, and 23 mM, respectively. To avoid any proton-induced effect on the optical properties (11,13–15), pH = 7.5 was used in all solutions.

Preparation of double-stranded DNA

Alexa-488 dye labeled 55 bps dsDNA (Alexa488-55bps-dsDNA) was made by annealing equimolar amounts of commercial 5′ Alexa-488 55-mer oligonucleotide to its complementary strand. The quality of the resulting DNA products was checked using polyacrylamide gel electrophoresis (data not shown).

DNA restriction reaction

Restriction reactions were executed in 20 μl reaction solutions (labeled RS1-4), containing NEBuffer 4, 0.23 mM TX100, 4.2 nM Alexa488-55bps-dsDNA, and 2 μl of the corresponding enzyme solution ES1-4. As a control, DNA solution RS5 was used, containing all components except enzyme. Restriction reactions were performed at 37°C for 1 h.

RESULTS

Protein adsorption to surfaces

Without special measures, proteins in low concentrations readily adhere to surfaces, since an excess of surface binding sites is available. As a consequence, protein concentrations in solution are at best approximate and experimentally derived parameters, such as affinity constants, are not reliable. To prevent protein adsorption, we tested treatment of glass surfaces by using TFOS to exposed silanol groups. In Fig. 1, eGFP adsorption of untreated (Fig. 1 *c*) and treated surfaces (Fig. 1 *b*) is shown. For ease of reference, Fig. 1 *a* shows a representative image of a standard cleaned and TFOS-passivated surface in the presence of HPLC water only. Color scales of Fig. 1, *a* and *b*,

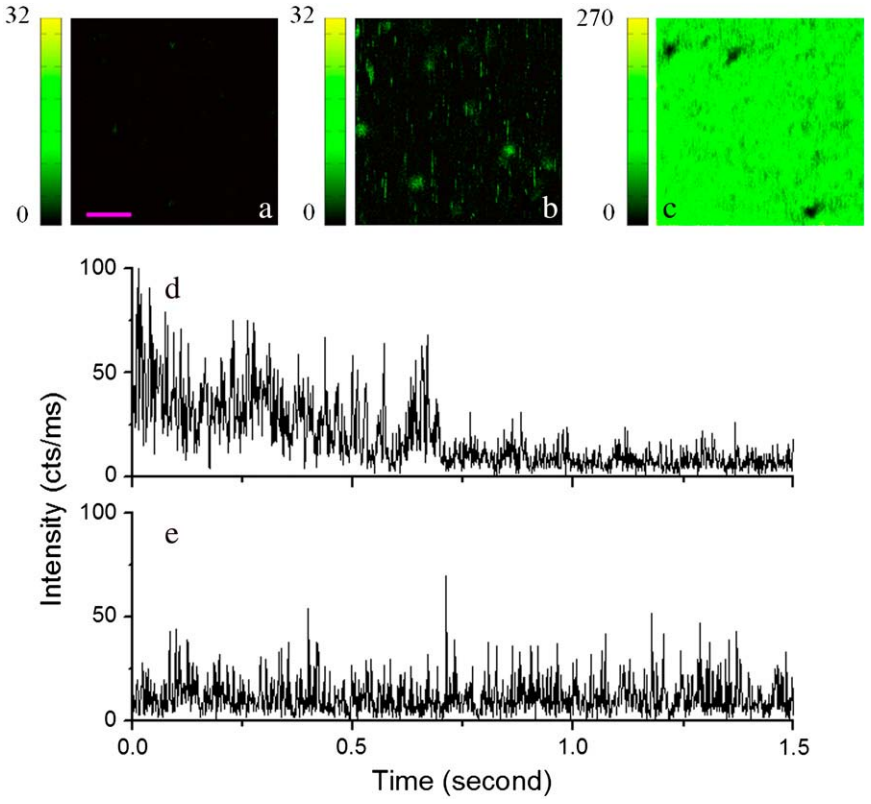

FIGURE 1 Protein adherence to surfaces is largely prevented by treating the surface with TFOS. Image size in *a*, *b*, and *c* is 9.3 × 9.3 μm^2, 256 × 256 pixels, dwell time 0.25 ms/pixel, excitation 7.9 $kW \cdot cm^{-2}$ at 488nm. (*a*) A representative image of a standard cleaned and TFOS-passivated surface in the presence of HPLC grade water only is shown. (*b*) A representative image of a TFOS-passivated surface after incubation with 10 nM eGFP in HPLC grade water is shown. (*c*) A representative image of a standard cleaned surface not passivated using TFOS after incubation of 10 nM eGFP in HPLC grade water is shown. Bar in *a* denotes 2 μm. Color scales in *a* and *b* are identical. In *d*, a representative intensity time trace of the glass-solution interface, as in *c* and *e* in solution, is shown.

are identical (counts per 250 μs). As can be clearly discerned, the adsorbed number-density of proteins at untreated glass is ~100 times higher than at treated glass. The data of Fig. 1 *b* are represented at a 10 times expanded color scale and only few adsorption events in an area of 9.3 × 9.3 μm^2 were observed. The maximum number of proteins per μm^2 in an 8 μL drop of 1 nM protein solution is ~800. Thus, the occupied surface area would in that case be <1%. Moreover, the (relative) immobility of surface adsorbed molecules results in rapid photobleaching of the fluorescence amplitude in a time trace (Fig. 1 *d*) as compared to that of molecules in solution (Fig. 1 *e*). Thus, we conclude that using TFOS-treated glass proteins do not stick to the surface.

All following experiments were performed using TFOS treated glass surfaces.

Loss of fluorescence of FPs in aqueous solutions

Despite the virtual absence of protein adsorption, gradual loss of fluorescent proteins from solution was still observed in our FCS experiments. In these experiments, fluorescence bursts were recorded during at least 500 s, resulting in a sequence of 50 × 10 s traces, which were subsequently calculated into an autocorrelation trace and fitted to a 3D-diffusion model (10) to extract molecular residence times and eGFP concentrations estimated from background-corrected amplitudes of the FCS curves. As shown in Fig. 2 *a*, eGFP fluorescent intensity in solution gradually decreased despite the virtual absence of protein adsorption. Molecular residence times are plotted in Fig. 2, *b* and *c*. From these measurements, it follows that the average residence time of eGFP (τ_D) is 149 ± 7 μs, which is in good agreement with Schenk et al. (19) and with the τ_D of R6G (see the Supplementary Material, Data S1). Similar results have been obtained using mStrawberry and mCherry (data not shown). In spectral time traces, spectral properties of diffusing species can be monitored. A typical spectral time trace of an eGFP solution (25,000 spectra with a total recording time of 5000 s) is presented in the Supplementary Material, Data S1, Fig. S1, *a* and *b*, respectively, which shows the decay in amplitude from spectral time traces and average spectra from subsequent time intervals. To test the potential influence of buffers used, similar experiments were conducted using eGFP dissolved in Tris-HCl buffer, HEPES-NaOH buffer, PBS buffer, and CP buffer. Fluorescence amplitude decreased over time in all instances (Fig. 3 *a*), whereas molecular residence times remained constant (Fig. 3 *b*). In contrast, 2.5 nM eGFP solution in 0.23 mM TX100 stably fluoresced until the experiment was stopped in 1 h. This clearly shows photobleaching is not the source of fluorescence loss of eGFP in the solution. Experiments for mStrawberry and mCherry instead of eGFP gave similar results (data not shown).

To test the potential influence of protein concentration in the stock solution, the concentration of the stock solution was increased to 10 nM. FCS measurements from fresh samples over a two-day time period revealed a similar loss of fluorescent protein signal in a few hours.

We conclude that despite the surface treatment using TFOS, a gradual decrease of fluorescence results under a wide range of conditions.

Activity of FPs in the presence of surfactant TX100

To develop a procedure to prevent loss of active fluorescent protein in solution, protein solutions were prepared containing varying concentrations of TX100, a well-known surfactant, ranging over 5 orders of magnitude from 0.23 μM to 23 mM., Five 100 μl 2.5 nM eGFP solutions were prepared (labeled FS1-5, see Methods), which were all stored in a dark polyurethane box under ambient conditions. Periodically, within a period of 34 days, 8 μl of each solution was used to monitor protein activity using FCS and spectral measurement. Apart from solution FS1, containing 2.3 μM of TX100, which completely lost fluorescence activity within a few hours, all solutions preserved fluorescence activity for more than 1 month (34 days). Fig. 4 *a* shows the spectra of FPs in FS2-5 (from the *top down*) on day 34 compared with that of a freshly prepared sample in 23 mM TX100 (*bottom*).

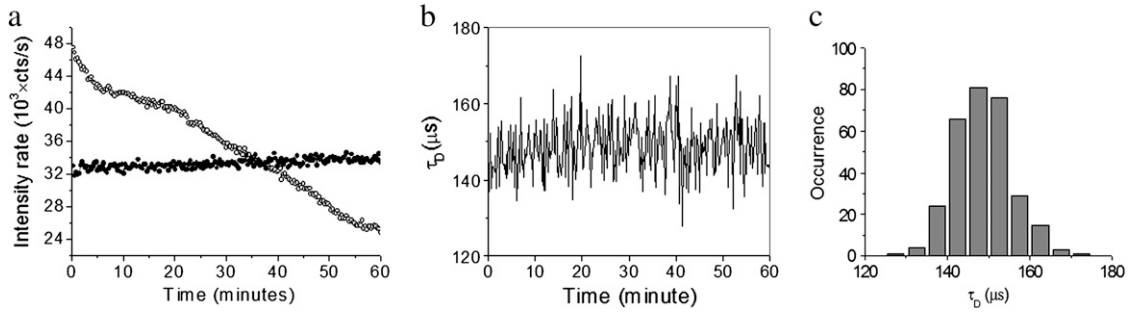

FIGURE 2 (*a*) Decrease of the fluorescence amplitude of a solution of 5 nM eGFP (*open circles*) in HPLC grade water and a stable fluorescence amplitude of a solution of 2.5 nM eGFP (*solid circles*) in 0.02% TX100. The excitation power was 16 kW·cm^{-2}. Excitation wavelength was 488 nm. (*b*) Simultaneously with the time trace in *a*, the residence time of eGFP in the focal volume, τ_D, was determined 360 times for 10 s intervals during 1 h. (*c*) The histogram of *b*. Average value and standard deviation of $\langle\tau_D\rangle$ = 149 ± 7 μs.

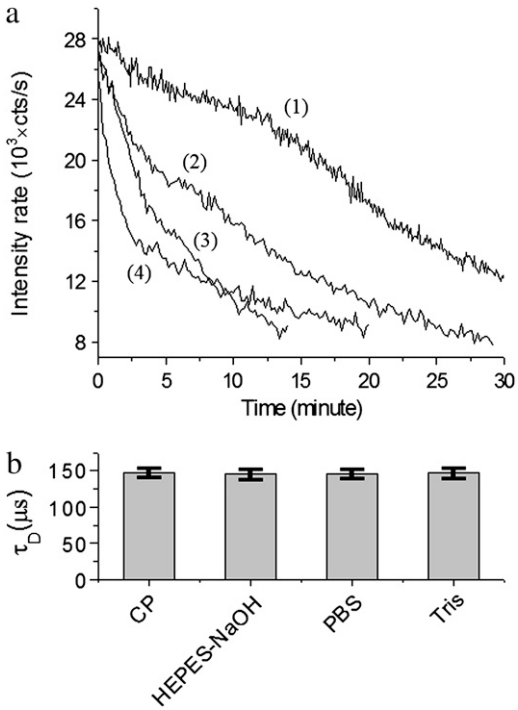

FIGURE 3 (*a*) Decrease in fluorescence amplitude of eGFP in FCS time traces in Tris-HCl buffer (1), HEPES-NaOH buffer (2), PBS buffer (3), and CP buffer (4). (*b*) The average and standard deviation of residence times, τ_D, of eGFP as obtained from the time traces in *a*.

The Raman band of water was used as an internal intensity marker. A constant ratio of fluorescent emission to this band was observed even after 34 days. A pairwise comparison of molecular residence times (Fig. 4 *b*) in the presence of four different TX100 concentrations showed near-identical behavior of fresh samples and samples stored for 34 days. In Fig. 4 *b*, it is shown that the highest concentration of TX100 (samples FFS5 and FS5 at 23 mM) results in a slight increase in molecular residence time, which could be the result of increased viscosity of the solution.

Identical experiments were conducted using mStrawberry and mCherry or using NP40 as surfactant instead of TX100, which all led to similar results (Fig. S2 in the Supplementary Material, Data S1).

The influence of high concentrations of other proteins was also tested. Nanomolar concentrations of eGFP were dissolved in buffer containing 0.1 mg/mL (1.47 μM) BSA. FCS measurements indicated that fluorescence activity of eGFP could be observed up to 72 h after sample preparation (data now shown), suggesting that BSA does indeed stabilize functionality but not as efficiently as TX100 or NP40.

Activity of restriction enzymes in the presence of surfactant TX100

The extraordinary long functionality lifetime of fluorescent proteins in dilute solutions (vide supra) in the presence of

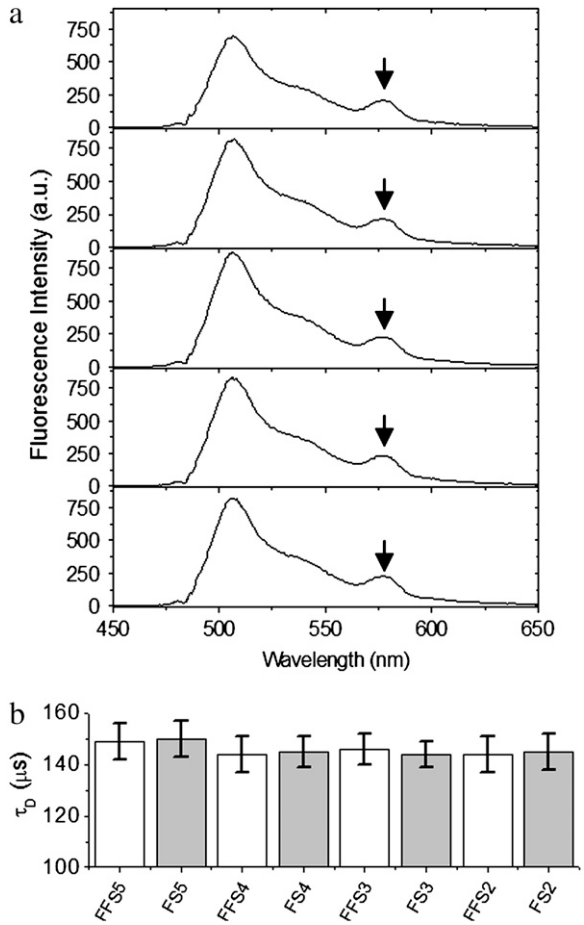

FIGURE 4 (*a*) Fluorescence emission spectra of 2.5 nM eGFP solutions FS2-5 (after 34 days under ambient condition) and FFS5 (freshly prepared), from top to bottom, by averaging 100 frames of spectra with a 200 ms acquisition time for each frame. Excitation used was 16 kW·cm^{-2} 488 nm laser. The water Raman band, indicated with arrows, served as internal intensity standard. (*b*) The residence times τ_D of eGFP as obtained from spectral time traces of eight different eGFP solutions FS2-5 (*shaded*) and FFS2-5 (*open*). Solutions FFS2-5 were freshly prepared. Histograms of *b* are shown in Fig. S3 (Supplementary Material, Data S1).

TX100 raises the question whether these observations also extend to other protein functionalities, such as the activity of restriction enzymes (20). To explore this, an Alexa-488 dye-labeled 55 basepairs dsDNA (Alexa488-55bps-dsDNA) containing a recognition site for *Fok*I was chosen (Fig. 5 *a*), which was positioned such that in the presence of active *Fok*I, a short Alexa-488 labeled double-stranded oligo would result. The expected rapid diffusion of the small oligo as measured by FCS was used as a reporter of the activity of the restriction enzyme *Fok*I.

Four 40 μl enzyme solutions, labeled ES1-4, were prepared containing 0.4 units/μl *Fok*I in buffer TEK without TX100 (ES1), or in buffer TEKT containing 0.23 mM TX100 (ES2), or containing 0.04 unit/μl *Fok*I in buffer TEK without TX100 (ES3) or buffer TEKT containing 0.23 mM TX100 (ES4). All *Fok*I solutions were prepared on ice and 2 μl of

each solution ES1-4 was immediately quantified with DNA restriction reactions (see Materials and Methods sections) by FCS ($t = 0$ h). The enzyme solutions ES1-4 were subsequently stored under ambient conditions, and enzyme activity was monitored by DNA restriction reactions and FCS over a period of 48 days. Fig. 5 *b* shows the average residence times of DNA molecules in all samples, which were obtained from 50 independent 10 s time traces. As can be observed, solutions containing low concentrations of *Fok*I (0.04 units/μl) in the absence of TX100 (ES3) completely lost activity within 72 h. However, in the presence of 0.23 mM TX100 (ES4), low concentrations of *Fok*I retained activity within 48 days. High concentrations (0.4 units/μl) of *Fok*I solution in the presence (ES2) and absence (ES1) of TX100 showed similar activity after 72 h, corroborating the results.

The same experiment was conducted using the restriction enzyme *Ava*I, for which similar results were obtained (data not shown). In conclusion, these results demonstrate that the addition of surfactant to solutions containing low protein concentrations prevents loss of protein activity.

DISCUSSION

We have used quantitative FCS to monitor the activity of proteins at dilute (nanomolar) concentrations in the presence and absence of surfactant. It was observed that the protein functionality lifetime at very dilute solutions was >1000-fold extended in the presence of surfactant TX100 and NP40. The effect of TX100 was achieved at a concentration of 23 μM and higher whereas no effect was observed when a concentration of 2.3 μM was used. The average aggregation number of TX100 in a TX100 micelle is ~147 (21).This implies that the average distance between TX100 micelles in a 23 μM solution is ~216 nm, whereas the average distance between proteins in a 1 nM solution is ~1.2 μm. These numbers show that the intermolecular distances are much larger than the molecular size of the surfactant (between 5 nm and 10 nm) and the protein (~4 nm for eGFP). The molecular residence time obtained from FCS directly shows that no long-lasting contact interaction takes place between the surfactant and the protein. An order of magnitude estimate of the result of the interaction of a single micelle (molecular mass $147 \times 628 \approx 90$ kDa) with a single eGFP molecule (molecular mass 30 kDa) would result in a molecular residence time of ~240 μs. This number is much different from the values we have obtained (Figs. 2–4) and clearly beyond the measurement error. Therefore, we conclude that the very large, 1000-fold effect of the detergent on the functionality lifetime of dilute protein in solution is achieved by another mechanism than direct contact interaction.

Structure and function of proteins are related to sub-nanometer scale interactions between protein side chains. These interactions are facilitated by a 3D arrangement of secondary structure motifs. The role of water in protein function and stability is well known (22). Water enables fast conformational changes, and clusters of water molecules have been found to form in or near hydrophobic cores in proteins (23). In very dilute protein samples, the environment is dominated by water, the behavior of which seems to be affected by the presence of surfactant (vide supra). Intramolecular H-bonds contribute greatly (23) to protein structure and stability and they are particularly effective in the absence of competing, free water molecules in the interior of the protein. On the other hand, interior water molecules do play an essential role in protein stability and they have much longer mean residence times than water molecules in bulk. Nevertheless, water molecules may dissociate and escape to the bulk as a result of or as part of a structural collapse (24) of a protein. The presence of free water molecules in the interior of proteins may lead to a so-called ''water attack'' (25,26) on the intramolecular protein hydrogen-bond network. It was shown by computer modeling and molecular dynamics simulations that such a water attack could lengthen the intramolecular H-bonds and rearrangements in the hydropho-

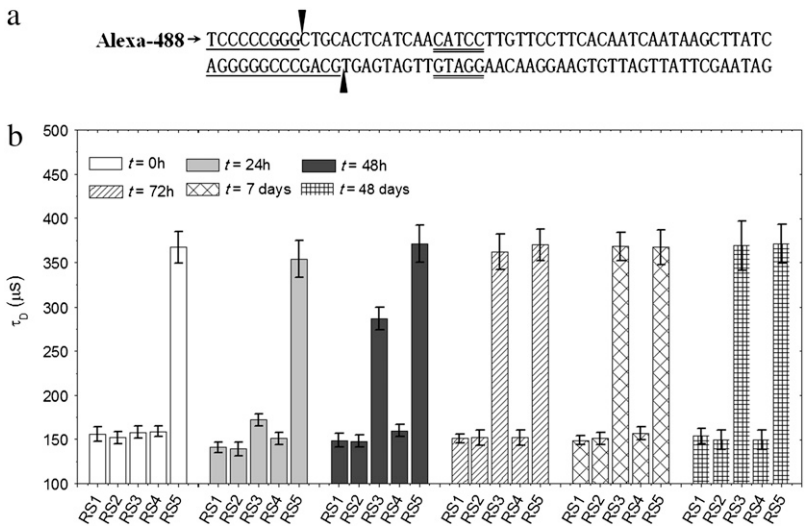

FIGURE 5 (*a*) 55bps-dsDNA was labeled with Alexa488. *Fok*I recognition sites are five basepairs (*double underlines*) in length. Cleavage positions on both strands are indicated by arrows. The restriction fragment containing the Alexa-488 dye is singly underlined. (*b*) The average residence times of Alexa488-DNA molecules from FCS measurements acquired at the indicated times during 48 days. Excitation for FCS measurements was 40 kW·cm^{-2} 488 nm laser. Reaction solutions RS1-4 contained 4.2 nM Alexa488-55bps-dsDNA, 0.8 units *Fok*I for RS1 and 2, and 0.08 units *Fok*I for RS3 and 4, respectively. The *Fok*I in the reaction solutions RS1–4 was obtained from the enzyme storage solutions ES1-4, respectively, which were stored at room temperature for the indicated times. ES2 and ES4 solutions contained 0.23 mM TX100. Solution RS5 contained similar components but no enzyme.

bic side chains to shield them from a more polar environment (25,26). Clearly, for eGFP in water to maintain its fluorescence, contacts between protein side chains and the Ser-Tyr-Gly chromophore should be shielded from hydration.

In bulk water, H-bond networks are governed by extremely fast dynamics, typically within an ~1 ps timescale (27,28). The dispersive forces of rapidly fluctuating constructive and destructive water H-bond networks may destabilize proteins by the repeated recurrence of destructive forces with a, in itself, small probability. A lower population of destructive H-bond network or a slower evolution of water H-bond network patterns may increase protein stability and in this way lengthen the functionality lifetime of a protein. We have provided ample evidence that TX100 (and other surfactants) increase the stability of protein functionality without directly interacting with the proteins. The question that arises is: how does TX100 (and other surfactants such as, e.g., NP40 and BSA) stabilize the functionality lifetime of soluble proteins?

The hydration shell of soluble proteins was proposed very early (29) to be of major influence on the functionality of proteins. Recent theoretical (30,31) as well as experimental (32) evidence indeed revealed that water molecules in the hydration shell exhibit anomalous behavior (30,31), such as subdiffusive translational diffusion and nonexponential orientation relaxation. The solvation dynamics of optical probes near the protein surface also show a slow decay over time (27,28). The solvation dynamics on surfactant micelles is known to be very slow as well and may even extend to a few nanoseconds (33). The general structure of nonionic micelles containing a dense hydrophobic core and hydrophilic polar headgroups on the surface is accompanied by a large effect on the dynamic properties of water molecules in the hydration shell of the micelle. Tight hydrogen bonding networks of water with polar headgroups may lead to solvation dynamics in the hydration shell of micelles that are slowed down by as much as 3 orders of magnitude.

Our observation that the protein functionality is ''remotely'' stabilized by the presence of nonionic surfactant micelles at a concentration higher than ~23 μM could reflect the slow water dynamics as a result of stable networks in the hydration shell of surfactants. This property effectively decreases the rate of occurrence of destructive dispersive water activity that competes with intramolecular protein hydrogen bonding and fixed water in proteins. An approximate average distance between TX100 micelles at a concentration of 23 μM is of the order of 200 nm. It follows that an influence of micelles on water dynamics extending over 100 nm would already lead to an, on average, continuous effect on water dynamics in solution. The size of FPs and restriction enzymes, geometrically typically some 4 nm plus their respective water shells, further contributes to the overlap of organized water structures in between micelles at a concentration of some tens of μM. At a 10 times lower TX100 concentration, coinciding with an approximate average distance of 450 nm, we have not observed an effect on the stabilization of protein functionality, suggesting that the influence of the micelle does not extend much farther than ~100 nm into the solvent.

Since using FCS we have directly measured relatively slow molecular diffusion, it is interesting to note that no effect on the protein diffusion rate was observed until ~23 mM of TX100. This concentration coincides with an approximate average distance of 22 nm between TX100 micelles. This agrees with our reasoning that the fast dynamics of the solvent are particularly affected at relatively low surfactant concentrations, whereas viscous effects of the surfactant influence the relatively slow molecular mobility at much higher concentration when the average distances between micelles become of ''molecular size''.

It follows that in the cells of organisms, where the concentration of proteins in the cytoplasm is between 100 and 250 mg/ml (34), protein functionality is stabilized in such a molecular crowded environment.

CONCLUSIONS

Quantitative fluorescence correlation spectroscopy of dilute, nanomolar solutions of fluorescent proteins and the restriction enzymes *Fok*I and *Ava*I reveals that the lifetime of protein functionality is extended at least 1000-fold in the presence of a sufficient concentration of surfactant molecules. We have argued that this effect is a result of the influence that micelles, and to a somewhat lesser degree the proteins themselves, have on the dynamic rearrangement of water hydrogen networks, limiting the occurrence of hydrogen bond networks in competition with the intramolecular protein networks that stabilize proteins. This model connects to ideas that have recently been proposed under the denominator ''water attack'' model (26) for protein destabilization. We feel that the reported effect at nanomolar dilutions of proteins contributes to a concept of ''biological water'' and could have a much broader range of applications. We also conclude that experiments in the single molecule optical regime can be carried out with fidelity in the presence of surfactant and passivated surfaces.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at www.biophysj.org.

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