Conformational Dynamics Govern the Free-Energy Landscape of a Membrane-Interacting Protein

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ABSTRACT: The equilibrium stabilities and the folding rates of membrane-bound proteins are determined by hydrophobic and polar intermolecular contacts with their environment as well as by intramolecular packing and conformational dynamics. The contributions of these factors, however, remain elusive and might vary considerably among proteins. Mistic from Bacillus subtilis is a particularly intriguing example of an α-helical protein that associates with membranes in spite of its unusual hydrophilicity. In micelles, Mistic is stabilized by hydrophobic and polar interactions with detergents, but it is unclear whether and how these intermolecular contacts are coupled to structural and dynamic adaptations of the protein itself. Here, we investigated the packing and the conformational dynamics of Mistic as functions of detergent headgroup chemistry and chain length, employing single-molecule Förster resonance energy transfer spectroscopy and time-resolved intrinsic tryptophan fluorescence spectroscopy. Surprisingly, in nonionic detergents, more effective hydrophobic burial and, thus, greater protein stability with increasing hydrophobic micellar thickness were accompanied by a gradual loosening of the helical bundle. By contrast, Mistic was found to assume a stable, compact fold in zwitterionic detergents that allowed faster dynamics on the nanosecond timescale. Thus, intramolecular packing per se is insufficient for conferring high protein stability; instead, enhanced nanosecond dynamics and, consequently, greater conformational entropy in the compact folded state account for Mistic’s high equilibrium stability and fast folding rates in zwitterionic micelles even at the expense of less effective hydrophobic burial.

INTRODUCTION

The folded states of membrane-associated proteins are stabilized by both intramolecular interactions upon packing and intermolecular interactions in the form of contacts with their membrane and water environment. For integral membrane proteins, which are firmly attached to lipid bilayers through very hydrophobic transmembrane segments, the contributions of these factors to protein stability are increasingly well understood. By contrast, the situation is less clear for membrane-interacting proteins that self-insert into the membrane and whose membrane attachment, therefore, cannot rely on pronounced hydrophobicity. Moreover, the role of protein dynamics and, hence, conformational entropy remains poorly understood for this class of proteins even though it could be crucial because of the large entropic changes that accompany coupled membrane binding and folding. A prominent example of a self-inserting protein is Mistic from Bacillus subtilis, a small membrane-associated protein essential for the regulation of bacterial biofilm formation. The protein contains 110 residues arranged into four α-helices. In spite of its polar, charged surface with a calculated pI of 4.5 and a net charge of −12 at pH 7, the helical bundle is tightly associated with membranes in vivo and membrane-mimetic systems such as detergent micelles in vitro. Lacking a signal recognition sequence, Mistic self-inserts into the bacterial membrane without the need for a translocon machinery. Remarkably, polar and charged residues are distributed all over the protein rather than being clustered in distinct regions that could represent extramembranous domains flanking a hydrophobic transmembrane domain, as is the case with canonical integral membrane proteins.

Consequently, the question arises as to the nature of the interactions that stabilize this unusually hydrophilic protein and promote its association with membranes and membrane mimics. When solubilized in detergent micelles, Mistic’s helical bundle is stabilized by both hydrophobic burial in the nonpolar micellar core and polar interactions with detergent headgroups, as borne out by protein-unfolding experiments at both the ensemble and the single-molecule levels. By contrast, the
secondary-structure content of Mistic is virtually unaffected by the nature of the detergent making up the micellar environment, as demonstrated by far-UV circular dichroism (CD) spectroscopy.13 Yet, it remains unclear whether and how changes in protein stability and intermolecular contacts with the complex, anisotropic membrane-mimetic environment are coupled to higher-order structural adaptations or to alterations in the conformational dynamics of the protein itself. To better understand the interrelationship among protein compaction, hydrophobic shielding, dynamics, and stability in different membrane mimics, we have investigated the effects of hydrophobic thickness and headgroup properties in various detergent micelles. To this end, we employed single-molecule Förster resonance energy transfer (smFRET) spectroscopy and time-resolved intrinsic fluorescence spectroscopy at the ensemble level to investigate protein compaction and conformational dynamics as functions of the micellar environment. Surprisingly, the degree of compaction was found to be inversely correlated with the protein’s equilibrium stability in nonionic micelles, whereas faster conformational dynamics and, as a corollary, greater conformational folded-state entropy in zwitterionic detergent micelles superseded poor hydrophobic shielding to afford high equilibrium stability and fast folding.

**RESULTS AND DISCUSSION**

**Structural adaptations of Mistic to its micellar environment.** We made use of smFRET confocal spectroscopy to probe the overall fold and, in particular, the degree of compaction of Mistic in response to changes in its micellar environment. To this end, we created a protein variant (Mistic$_{3,110}$) that was site-specifically labeled with acceptor (ATTO647N) and donor (ATTO532) dyes at positions 3 and 110, respectively (Figure 1a). To assess the influence of hydrophobic thickness, we used alkyl maltoside detergents ranging in chain length from C8 to C12 (Figure 1b), that is, n-octyl-β-D-maltopyranoside (OM), n-nonyl-β-D-maltopyranoside (NM), n-decyl-β-D-maltopyranoside (DM), n-undecyl-β-D-maltopyranoside (UM), and n-dodecyl-β-D-maltopyranoside (DDM). Moreover, to explore the influence of polar contacts

![Figure 1](image-url)

*Figure 1.* (a) Ribbon representation of Mistic$_{3,110}$ based on an NMR structure of Mistic (PDB 1YGM) in the presence of LDAO with extrinsic acceptor (red, ATTO647N) and donor (green, ATTO532) dyes attached to positions 3 and 110, respectively, as well as the native Trp13 residue (blue). (b) Chemical structures of detergents used.

![Figure 2](image-url)

*Figure 2.* smFRET spectroscopy of Mistic$_{3,110}$ in different detergent micelles. (a) Histograms of FRET efficiency, $E$, of Mistic$_{3,110}$ in alkyl maltosides with normal-distribution fits (black lines). (b) $E$ histograms of Mistic$_{3,110}$ in DPC and LDAO with normal-distribution fits (black lines). (c) Peak positions, $E_{max}$, and distribution widths represented as FWHM (bars), of the histograms in panels a and b versus number of carbon atoms in the detergent alkyl chain, $n_C$; ∼5 pM Mistic$_{3,110}$ in 1 mM micellar detergent at 24 °C; buffer: 50 mM tris(hydroxymethyl)-aminomethane (Tris), 50 mM NaCl, pH 7.4.

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and hydration, we employed C12 detergents with amine oxide and phosphocholine headgroups (Figure 1b), that is, lauryldimethylamine N-oxide (LDAO) and n-dodecylphosphocholine (DPC), respectively. Each detergent was used at a concentration of at least 1 mM above its critical micellar concentration (CMC; cf. Table S1) to ensure the presence of micelles.

Fluorescence bursts from a large number of individual molecules were analyzed to generate histograms of FRET efficiencies, $E$ (Figure 2a,b). For the homologous alkyl maldietides series, all histograms revealed a unimodal distribution that shifted monotonically from intermediate $E$ series, all histograms revealed a unimodal distribution that chain detergents (i.e., DDM) to higher $E$ micelles.

Conformational heterogeneity as the width is in large excess of that expected for shot noise alone (Figure S1a). Conversely, for short-chain alkyl maldietides (i.e., OM), the much narrower distribution anticorrelates with protein stability, as the molar Gibbs free-energy change upon unfolding, $\Delta G^\circ$ (H2O), drastically decreases on going from DDM ($\Delta G^\circ$ (H2O) = 16.3 kJ/mol) to OM ($\Delta G^\circ$ (H2O) < 7 kJ/mol).

By contrast, in DPC and LDAO, FRET distributions were shifted to higher $E_{\text{max}}$ values, with mean interdye distances of 4.7 nm in DPC and 4.5 nm in LDAO. This indicates that Mistic underwent considerable compaction when solubilized in zwitterionic detergents. Moreover, the degrees of broadening in DPC and LDAO were similar to that in OM (Figure S1b), indicating comparable levels of compaction and conformational heterogeneity. In striking contrast with the case of OM, however, protein compaction in zwitterionic micelles goes in hand with an increase in stability, which amounts to $\Delta G^\circ$ (H2O) = 18.5 kJ/mol in DPC and even results in resistance toward complete unfolding in LDAO.13,15 Two other protein variants fluorescently labeled at positions 3 and 88 (Mistic 3,88, Figure S3) and at positions 30 and 110 (Mistic 30,110, Figure S4) revealed the same dependences on detergent chain length and headgroup chemistry, thereby demonstrating that the above findings were not specific to the Mistic 3,110 variant but reflected a global, overall compaction of the micelle-embedded protein with decreasing chain length in nonionic alkyl maldietides and upon immersion in zwitterionic detergents.

In a next set of experiments, we followed intrinsic protein fluorescence of the single tryptophan residue at position 13 in Mistic’s first helix (Figure 1a) to study protein dynamics in the vicinity of the lone tryptophan residue on the nanosecond timescale. To relate protein compaction to the size of the protein/detergent complex, we performed time-resolved fluorescence anisotropy measurements to probe the rotational diffusion of Trp13. Experimental anisotropy decays were fitted using biexponential functions reflecting two rotational correlation times (Figure S5, Table S3). Whereas the faster of the two rotational correlation times, $\theta_{\text{fast}}$, is known to reflect the segmental mobility of the fluorophore, the slower one, $\theta_{\text{slow}}$, reports on the rotational diffusion of the protein/detergent complex (eq S5).19 To enhance the robustness of the fits, we reduced the number of floating parameters by fixing $\theta_{\text{fast}}$, at 0.5 ns, a value typical of single-tryptophan proteins.20 Accordingly, we used $\theta_{\text{slow}}$ to estimate the hydrodynamic volume of the protein/detergent complex, $V$, that is, the volume of a (hypothetical) sphere that would rotate with the same correlation time (eq S6). The volumes thus estimated (Figure 3a, Table S3) are in reasonable agreement with the values expected for spherical protein/detergent complexes with radii of 2–3 nm.

Overall, $V$ reproduced the trends found for protein compaction. In alkyl maldietides, $V$ monotonically decreased with decreasing chain length with values of 90 and 60 nm$^3$ for DDM and OM, respectively. This dependence of the

Figure 3. Hydrodynamic volume of Mistic/detergent complexes and accessibility to acrylamide and nanosecond relaxation kinetics of Mistic’s Trp13 in different detergent micelles. (a) Slow rotational correlation time, $\theta_{\text{slow}}$, and corresponding hydrodynamic volume, $V$ (eq S6), from anisotropy decays (Figure S5) versus number of carbon atoms in the detergent alkyl chain, $n_c$. (b) Bimolecular quenching rate constant, $k_q$, from time-resolved fluorescence quenching (Figure S6) plotted against the number of carbon atoms in the detergent alkyl chain, $n_c$. (c) Correlation time within 0–15 ns, $\tau_{\text{cf}}$, for dipolar relaxation (Figure S7) versus $n_c$, with data in OM, DM, DDM, and LDAO taken from reference 26. Mean values and standard deviations (error bars, often smaller than symbols) from three experiments; 4 μM Mistic in 5 mM micellar detergent at 20 °C; buffer: 50 mM Tris, 50 mM NaCl, pH 7.4.
Conformational dynamics of the protein/detergent complex on the nanosecond timescale. The absence of simple structural predictors prompted us to dissect the role of dynamic processes in determining protein stability in membrane-mimetic environments. To this end, we measured time-resolved emission spectra (TRES) of the native Trp13 chromophore of Mistic to extract time-dependent fluorescence shifts (TDFs). These serve as sensitive metrics of dipolar relaxation and allow determination of the correlation time, \( \tau_{cor} \) (eqs S14–S15), which reports on the nanosecond fluctuations of the protein/detergent complex around the tryptophan chromophore.\(^{26}\) Using \( \tau_{cor} \), one can thus infer the roughness of the underlying energy landscape of the folded state of the protein embedded in the respective micellar environment. For Mistic, we constructed TRES (eqs S10–S12) and followed the temporal progression of the average emission energy (eq S13, Figure S7). Differences in detergent headgroup polarity manifested markedly in the nanosecond relaxation dynamics. With values of 5.7–6.5 ns, \( \tau_{cor} \) was similar in all alkyl maltosides irrespective of chain length, whereas DPC and LDAO exhibited considerably smaller values of 4.9 ns and 3.7 ns, respectively (Figure 3c, Table S5). Strikingly, the differences in \( \tau_{cor} \) among DDM, DPC, and LDAO observed on the nanosecond timescale parallel the differences among the same three detergents previously found for the folding dynamics of Mistic on the microsecond timescale, with folding time constants of \( \sim 700 \), \( \sim 70 \), and \( \sim 30 \) \( \mu s \) for DDM, DPC, and LDAO, respectively.\(^{17} \) This correlation suggests that the large-scale folding dynamics of Mistic in the microsecond regime may be coupled to small-scale conformational dynamics in the nanosecond regime.\(^{26} \) Molecular dynamics simulations of Mistic in LDAO micelles show a drift in the arrangement of the four protein helices that occurs on a timescale of 20–30 ns without significant changes in the secondary structure.\(^{27} \) Although the starting point in these simulations is different from the photoexcited state probed here, similar timescales imply that rigid-body motions of the entire helical segments might explain the nanosecond relaxation dynamics of Mistic.

Taken together, we found Mistic to assume a compact yet highly dynamic fold in zwitterionic micelles. This can explain its high thermodynamic stability\(^{15} \) and fast folding rate\(^{15} \) in zwitterionic micelles because such pronounced residual dynamics in the folded state should considerably reduce the loss in conformational entropy incurred upon folding.\(^{26} \) Changes in the residual entropy of folded proteins have been recognized as major determinants of ligand-binding affinity.\(^{28–31} \) The present results then imply that for membrane-interacting proteins the conformational entropy of the folded state similarly depends on interactions with the membrane or membrane-mimetic environment, thereby suggesting that it acts as a major driving force in stabilizing the native fold.

### CONCLUSIONS

A complementary suite of fluorescence-based methods allowed us to draw a detailed picture of how the conformational dynamics of the helical-bundle protein Mistic in different membrane mimics are coupled to structural adaptations of the protein, its intermolecular contacts with the complex, anisotropic solvent, as well as its equilibrium stability and folding kinetics. In nonionic detergents, Mistic achieves higher stability with increasing micellar thickness by a gradual loosening of its helical bundle that results in greater structural heterogeneity. In the language of simple free-energy landscapes (Figure 4), this corresponds to a transition from a narrow, moderately rugged folded-state minimum (Figure 4a) to a broader, heterogeneous conformational distribution with significant free-energy barriers (Figure 4b). By contrast, in zwitterionic micelles, which offer less effective shielding from water but promote richer polar contacts with detergent headgroups, Mistic assumes a stable and compact yet dynamic fold with reduced free-energy barriers within a narrow folded-
state minimum (Figure 4c). Because Mistic exhibits a similar equilibrium unfolding behavior in the anionic detergent sodium dodecyl sulfate to that in LDAO and DPC, it appears very likely that the situation observed here for zwitterionic detergents is representative of ionic detergents as well. Furthermore, our results underline the extraordinary relevance of electrostatic interactions between the highly polar surface of Mistic and (zwitter)ionic headgroups in stabilizing this unusual protein within a membrane-mimetic environment. Future experiments on reconstituted proteoliposomes of various lipid compositions will shed light on how the membrane-associated state of Mistic is a protein with high equilibrium stability and fast folding kinetics.

### EXPERIMENTAL SECTION

#### Materials

All chemicals were purchased with the highest purity available. OM, NM, DM, UM, DDM, and DPC were from Glycon Biochemicals (Luckenwalde, Germany) or Anatrace (Maumee, USA). LDAO was from Anatrace, Tris from Carl Roth (Karlsruhe, Germany), NaCl (AnalaR Normapur) from VWR (Darmstadt, Germany) or Carl Roth, 1,4-dithiothreitol (DTT) from Sigma–Aldrich (Steinheim, Germany), and N-propargyl-L-lysine (PrK) from SiChem (Bremen, Germany). ATTOS32 maleimide and ATTO647N azide were from Atto-Tec (Siegen, Germany).

#### Protein preparation

Production and purification of the wild-type protein and detergent exchange were performed as described elsewhere. Briefly, Mistic was recombinantly produced with a hexahistidine tag in Escherichia coli BL21 (DE3) cells using a pET-30 EK/LIC expression vector (Merck, Darmstadt, Germany). Cells were lysed by sonication, cell debris was removed by centrifugation, and the tagged protein was purified in LDAO by immobilized-metal ion affinity (IMAC) chromatography. The tag was removed by digestion with enterokinase followed by anion-exchange chromatography (AIX). An additional IMAC step was performed to collect tag-free protein in the flow-through. Detergent was exchanged by AIX and size-exclusion chromatography (SEC).

For smFRET experiments, three protein variants (i.e., Mistic$_{120}^{30}$, Mistic$_{120}^{88}$, and Mistic$_{120}^{88}$) were engineered that contained the unnatural amino acid PrK and a unique Cys incorporated at residues 3 and 110, 30 and 110, or 3 and 88, respectively. Recombinant protein production was performed in E. coli using the pEvol vector system (pEvol PyrRS) from Methanosarcina mazei for incorporation of PrK. After purification following published procedures, the protein variants were orthogonally labeled with azide-functionalized acceptor (ATTO647N) and maleimide-functionalized donor (ATTO532) fluorophores using azide–azide click chemistry and thiol–maleimide coupling. Labeled proteins were separated from unbound dyes by SEC.

#### smFRET spectroscopy

Observations of single-molecule fluorescence were made using a custom-built dual-color, dual-polarization epifluorescence confocal setup based on an inverted microscope (Eclipse Ti-E, Nikon, Tokyo, Japan), which was equipped with a high-numerical aperture water immersion objective (CFI Plan Apo WI 60x, NA 1.2, Nikon, Tokyo, Japan) and two linearly polarized 530-nm and 640-nm picosecond pulsed laser sources (LDH-P-FA-530L and LDH-D-C-640, both from PicosQuant, Berlin, Germany) for pulsed interleaved excitation (PIE). Emitted donor and acceptor fluorescence were detected by single-photon-counting avalanche diodes (r-SPADs, PicosQuant) coupled to a time-correlated single-photon-counting (TCSPC) module (HydAmp 400, PicosQuant), as detailed elsewhere. Measurements were performed at 24 °C on freely diffusing molecules. Labeled Mistic variants were diluted from stock solutions into buffer (50 mM Tris, 50 mM NaCl, pH 7.4) containing the respective detergent at a micellar concentration of 1 mM (i.e., CM + 1 mM; Table S1) to a final protein concentration of ~5 pM to obtain burst rates under single-molecule conditions. The illumination power was 110 μW for excitation of the donor dye and 90 μW for direct excitation of the acceptor dye.

Data analysis was performed with custom-written Matlab scripts (Mathworks, Natick, MA, USA) following procedures described previously. Briefly, after identification of bursts with a search algorithm (using maximum interphoton time of 50 μs, minimum total number of 100 photons after background correction, and a Lee filter of 4), histograms of FRET efficiency, $E_b$, with bin widths of 0.03 were constructed from bursts exhibiting a stoichiometry ratio of $S = 0.35 - 0.7$ and an alternating laser excitation–two-channel kernel-based density distribution estimator (ALEX-2CDE) score $<10$. Additionally, an asymmetric burst filter was applied using a channel-asymmetry time of $<50 μs$. $E_b$ values were corrected for background, direct acceptor excitation, channel cross-talk, as well as differences in detector efficiencies and quantum yields between the dyes, as described previously. Apparent FRET efficiencies, $E_{app}$, used in probability-distribution analysis (PDA) were calculated without applying the aforementioned corrections. $E_b$ histogram distributions were fitted with normal or log-normal distributions to extract $E_b$ values at peak positions, $E_{max}$ as well as the full-width at half-maximum height (FWHM). Distances, $r$, were estimated on the basis of the Förster equation, $r = R_0 \times (1/E - 1)^{-1/6}$, where $R_0$ is the Förster distance of the ATTO532/ATTO647N FRET pair ($R_0 = 5.9$ nm), with the assumption that the fluorophores can freely rotate at the labeling site. Estimated histogram shot-noise-limited distribution widths (see Figure S1) were generated by simulation using static PDA. Recurrence analysis of single particles (RASP) (see Figure S2) was performed as previously described.

#### Tryptophan Fluorescence Spectroscopy

General. Samples contained 4 μM Mistic in detergent at a micellar concentration of 5 mM (Table S1) in buffer (50 mM Tris, 50 mM NaCl, pH 7.4). 5 mM DTT was freshly added prior to
measurements to prevent dimerization through the lone cysteine residue at position 3. For each detergent, three samples were prepared and measured independently. Experiments were performed at 20 °C in a 3 mm × 3 mm quartz glass cuvette (Hellma, Müllheim, Germany) on a FluoTime 300 spectrometer (PicoQuant), equipped with a PLS 280 pulsed laser diode (PicoQuant) emitting at (281 ± 5) nm and a 334-nm short-pass filter (LOT Quantum Design, Darmstadt, Germany). The emission bandwidth was set to 10 nm. Fluorescence emission decays were recorded at 355 nm with a detection wavelength set to the excitation wavelength. All measurements were performed at the otherwise optimal settings. Instrument response functions were obtained by measuring light scattered by Ludox dispersion with the excitation or emission polarization as detailed above. Steady-state emission spectra were determined from fluorescence decays with excitation polarized at 0° and 90° (i.e., the magic angle), respectively, if not stated otherwise. Instrument response functions were obtained by measuring light scattered by Ludox dispersion with the excitation wavelength set to the excitation wavelength. All samples exhibited low optical densities of ~0.1 or less, thus avoiding issues due to the inner-filter effect. Analysis of fluorescence emission and anisotropy decays was performed with the software FluoFit (PicoQuant). Models and equations used are described in the Supporting Information (eqs S1–S5).

In all cases, fluorescence decays were fitted very well on the assumption of three lifetime components and, therefore, were parameterized using triexponential fits (eq S1). Fluorescence decays at an emission wavelength of (355 ± 5) nm were analyzed to extract intensity-weighted average lifetimes, 〈τ〉 (eq S2).31,42 The latter were similar for all alkyl maltoside detergents, with values of ~4.4 ns, but slightly higher for zwitterionic detergents, with 4.8 ns and 4.7 ns for LDAO and DPC, respectively (Table S6).

Time-resolved anisotropy. Anisotropy decays were determined from fluorescence decays with excitation polarized at 0° and emission polarized at either 0° or 90° for 300 s each (eqs S3 and S4).

Time-resolved quenching. Fluorescence decays were recorded in the presence of increasing acrylamide concentration ranging from 0 to 0.25 M with a bin size of 50 ps (rather than 25 ps as for the other experiments, to reduce the total measurement time in titration series), one scan per acrylamide concentration, and in the absence of DTT. In the presence of DTT, the quenching efficiency showed an unexpected downward curvature when plotted against quencher concentration. As experiments with the water-soluble Trp analogue, N-acetyl-l-triptophanamide (NATA), showed the same trend (data not shown), we concluded that this unusual behavior was caused by DTT, which therefore was omitted from all experiments involving acrylamide quenching. As the results from time-resolved quenching were in excellent agreement with those from steady-state fluorescence spectroscopy and equilibrium denaturant-shielding measurements performed in the presence of DTT,15 it can be concluded that dimerization was negligible in the absence of DTT during the time course of the time-resolved quenching experiments.

Time-resolved emission spectra. TRES were determined, analyzed, and interpreted as detailed elsewhere.26 Briefly, fluorescence decays were recorded in the wavelength range of 310–450 nm with a step size of 5 nm, a bin size of 50 ps, and a data-acquisition time of 300 s per wavelength. Other settings were as detailed above. Steady-state emission spectra were recorded across the same wavelength range with the same spectral resolution, an integration time of 1 s per data point, and excitation and emission polarized at 90° and 0°, respectively. Each sample was scanned five times, and steady-state spectra were further processed by averaging and subtracting the corresponding blank spectrum of a protein-free detergent solution.

[ASSOCIATED CONTENT]

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01609.

Theoretical background with models and equations used for analysis of time-resolved fluorescence data (eqs S1–S5), supporting figures showing additional smFRET data for Mistic3,10 from shot-noise (Figure S1) and recurrence analysis (Figure S2), smFRET results for other labeled Mistic variants (Figures S3 and S4), time-resolved anisotropy decays (Figure S5), time-resolved fluorescence quenching curves (Figure S6), and dipolar relaxation of Trp13 fluorescence (Figure S7), as well as supporting tables with CMCs of detergents used (Table S1) and numerical values from smFRET and tryptophan fluorescence experiments (Tables S2–S6) (PDF)

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Notes
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