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Ligand Binding Transmits Conformational Changes across the Membrane-Spanning Region to the Intracellular Side of the 5-HT_3 Serotonin Receptor

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The serotonin-gated 5-HT_3 receptor (5-HT_3R) is a member of the Cys-loop receptor family, which includes the nicotinic acetylcholine, $GABA_A$, and glycine receptors.[1–3] All these receptors open an ion channel upon binding a specific neurotransmitter and thereby mediate fast signal transduction across synapses in the nervous system. Besides their central roles in normal neuronal signaling, mutations in their genes cause severe neurological diseases such as epilepsy, hyperekplexia, and congenital myasthenia, to mention but a few.[4,5] Biochemical and biophysical investigations have revealed the 5-HT_3R to be a homopentamer; each of its subunits comprises a large extracellular N-terminal ligand-binding domain[6–11] followed by four transmembrane segments (Figure 1A and B).[12,13] The question of how agonist binding leads to channel opening, that is, transmembrane signaling, and which conformational rearrangements are involved, remains unresolved because of the absence of high-resolution crystal structures of the open and closed channel states. However, agonist binding must be coupled to the opening of the ion channel. In addition to the transmembrane region M2, which contains the receptor gate, several other segments, including M4, move as a consequence of conformational changes induced by agonist-mediated re-

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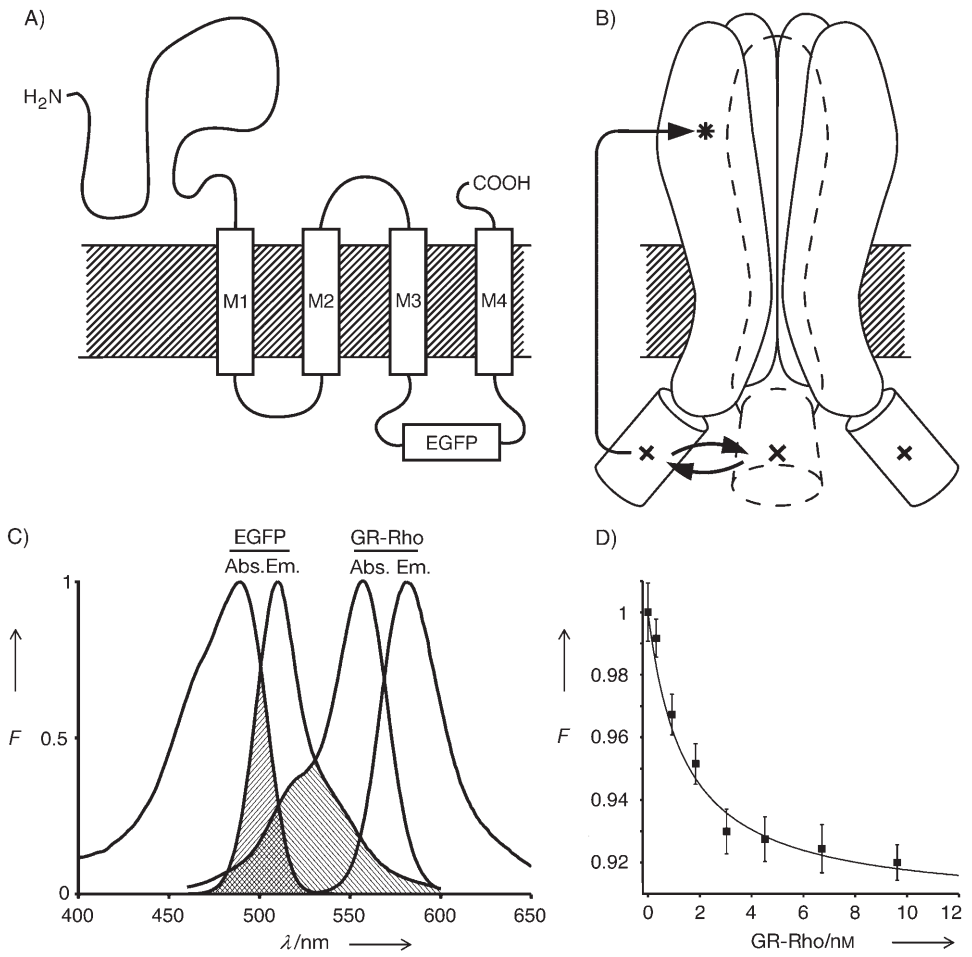

Figure 1. Spectral properties of detergent-solubilized EGFP-labeled 5-HT_3R and quantitative FRET-based evaluations of receptor interactions with a fluorescent antagonist. A) Membrane topology of a single 5-HT_3R subunit showing the position of the EGFP molecule inserted in the cytoplasmic M3–M4 loop. B) Structural model of the homopentameric 5-HT_3R depicting the EGFP molecules as cylindrical structures. One 5-HT_3R subunit is drawn with dashed lines for clarity. Homo-FRET occurs between neighboring subunits, while hetero-FRET takes place between the EGFP moieties and a fluorescently labeled ligand (arrows). The rhodamine label of the antagonist, GR-Rho, used in the study is represented by a star, and the EGFP cores are indicated by crosses. C) Absorbance and fluorescence spectra of detergent-solubilized 5-HT_3R–EGFP and antagonist GR-Rho showing spectral overlaps (gray shaded regions) that are favorable for both homo- and hetero-FRET. Assuming the refractive index of the medium between the donor and acceptor to be equal to that of water, the orientation factor κ^2 is $^2/_3$, and the donor quantum yield is 0.6, the calculated Förster distances R_0 for homo- and hetero-FRET are 40.6 and 47.7 Å, respectively. D) Relative fluorescence intensity of the EGFP-labeled receptor at different concentrations of GR-Rho. The EGFP fluorescence emission intensity of detergent-solubilized receptor was measured 3 min after incubation with the ligand at 512 nm.

ceptor activation. Evidence for this has been provided by studies on the nicotinic acetylcholine receptor.[14–17]

We have explored new strategies for facile optical monitoring of ligand-gated ion-channel activation by detecting agonist-induced dynamic rearrangements in the protein structure. Using the 5-HT_3R, we show that extracellular binding of an agonist transmits a conformational change to the intracellular side of the receptor. This study was made possible by the insertion of enhanced green fluorescent protein (EGFP) into the cytoplasmic M3–M4 loop of each of the five receptor subunits (Figure 1 A). This method allowed the formation of ionotropic receptors with intrinsic fluorophores in a homopentameric arrangement (Figure 1 B). The fusion of GFP to ligand-gated receptors has already been used for noninvasive studies of expression, trafficking, and function.[18–23] As a novel strategy, we demonstrate the use of EGFP as a structural probe for sensing ligand binding by fluorescence resonance energy transfer (FRET) and show conformational changes induced by ligand activation by using homo-FRET. Here we show that ligand binding and channel activity in 5-HT_3R–EGFP remain fully functional, as for the 5-HT_3R variant that was fused to the enhanced cyan-fluorescent protein (ECFP), as reported elsewhere.[18] Our approach delivers quantitative data on 5-HT_3R activation, exhibits potency as a generic method for probing the structure and function of mutants and subtypes of ionotropic receptors, and therefore has potential for screening for therapeutic substances.

Radioligand-binding assays and whole-cell patch-clamp measurements revealed the ligand binding and channel activities of 5HT_3R–EGFP (Table 1) to be similar to those of the wild-type 5HT_3R.[18] This is an important result because it shows that the insertion of five EGFP molecules into 5HT_3R leaves the biological function of the receptor unchanged. The optical properties of 5HT_3R–EGFP were investigated by using detergent-solubilized plasma membranes that were isolated from HEK293 cells.

Firstly, using FRET, we show that the EGFP probes can act as fluorescent donors for measuring ligand binding to the receptor. The fluorescence intensity of EGFP was followed at 512 nm with increasing concentrations of the rhodamine-labeled, 5-HT_3R-specific antagonist GR-Rho (as acceptor); dose-dependent quenching of EGFP fluorescence was observed (Figure 1 B and C). GR-Rho did not induce quenching when the receptor was preincubated with an excess of the nonfluorescent competitive ligand quipazine (1 μM). This proved that the observed FRET was due to specific binding of GR-Rho to the receptor. From the FRET assay, a dissociation constant (K_d) of 1.4 nM was calculated, which is identical to the K_d value obtained from radioligand binding (Table 1).

Table 1. Functional characterization of 5-HT_3R–EGFP.[a]

	GR65630	GR-Rho		*m*CPBG	
	K_d [nM]	EC_{50} [nM]	K_d [nM]	EC_{50} [μM]	Hill coefficient
RLB/electrophysiology	0.7 ± 0.2	42 ± 6	1.2 ± 0.5	0.66 ± 0.08	1.4 ± 0.2
EGFP FRET	–	–	1.4 ± 0.3	0.5 ± 0.2	0.8 ± 0.3

[a] Dissociation constants (K_d) and channel activation were determined by radioligand binding (RLB) and whole-cell electrophysiology on transiently transfected HEK293 cells; data are compared to results obtained by in vitro EGFP fluorescence-intensity analysis. Saturation-binding experiments were performed with the radioactive ligand [^{3}H]GR65630. The K_d of the rhodamine-labeled antagonist, GR-Rho, was calculated by using the EC_{50} value obtained by competitive displacement of [^{3}H]GR65630 (0.8 nM). Ligand concentration-dependent FRET recordings between GR-Rho and the intrinsic EGFP labels of the receptor (Figure 1D) yielded a K_d value that was comparable to the value obtained by RLB. Agonist-mediated 5-HT_3R–EGFP channel activations that were acquired from whole cell patch-clamp experiments delivered an EC_{50} value for *m*CPBG that was in the range obtained with the wild-type receptor.[18] It is in agreement with the EC_{50} determined for the *m*CPBG concentration-dependent fluorescence intensity increase that was measured with detergent-solubilized 5-HT_3R–EGFP (see Figure 3).

Close proximity between neighboring EGFPs in the cytoplasmic region of the homopentameric 5-HT_3R–EGFP led to partial quenching of their fluorescence by homo-FRET. This was due to a sufficiently large overlap between their absorption and fluorescence spectra with a characteristic Förster distance of $R_0 = 40.6$ Å (Figure 1C). In turn, intracellular conformational changes in the receptor could be sensed by homo-FRET if they modulated the distances between neighboring EGFPs. This was first tested by monitoring the EGFP fluorescence of 5-HT_3R–EGFP during temperature-induced unfolding of the receptor. Circular dichroism (CD) experiments on purified 5-HT_3R showed an S-shaped thermotropic change of the ellipticity at 222 nm with a transition temperature ($T_t \approx 57.5\,^\circ$C; Figure 2A) that is clearly below that of GFP ($T_t = 78\,^\circ$C) and distinctly lower than the cloud point of the detergent $C_{12}E_9$ (88 °C).[24,25] This indicates that the CD experiments were not influenced by structural transitions of the detergent. In fact, the fluorescence intensity of 5-HT_3R–EGFP showed an S-shaped intensity temperature profile that had an inflection at $T_t \approx 57.5\,^\circ$C; this coincides with the CD-temperature profile of protein unfolding (Figure 2B). It is reasonable to assume that the unfolding of the receptor is accompanied by an increase in the average distances between the five EGFP molecules. This in turn reduces the homo-FRET between the EGFP molecules and thus explains the observed fluorescence intensity increase.

Our interpretation is corroborated by measurements of the fluorescence anisotropy (r) of the receptor and of free EGFP (Figure 2C). The r values of both components are nearly identical between 20 and 40 °C—a temperature range over which the receptor was shown by CD to have a stable structure. This is surprising at first glance because one would expect a distinctly higher r value for the detergent-solubilized receptor than for the purified recombinant EGFP (rEGFP) due to its 17-times higher molecular mass. In fact, such a decreased fluorescence anisotropy is due to high local concentrations of the five EGFP fluorophores in 5-HT_3R–EGFP, which lead to homo-FRET that is accompanied by a certain loss of polarization (as explained by Weber for concentrated fluorophore solutions).[26]

Above 40 °C, we observed differing tendencies for the fluorescence anisotropies. While r for EGFP continued to decrease slightly with increasing temperature, r for 5-HT_3R–EGFP increased substantially with an S-shaped profile that shows an inflection at $T_t \approx 59.5\,^\circ$C, which is close to the melting temperature of the receptor (Figure 2C). This clearly correlates with the fluorescence intensity increase (Figure 2B) and can be explained by an increase of the fluorescence lifetime of the EGFP molecules in unfolded 5-HT_3R–EGFP.

Given these findings, we conclude that the relative proximity

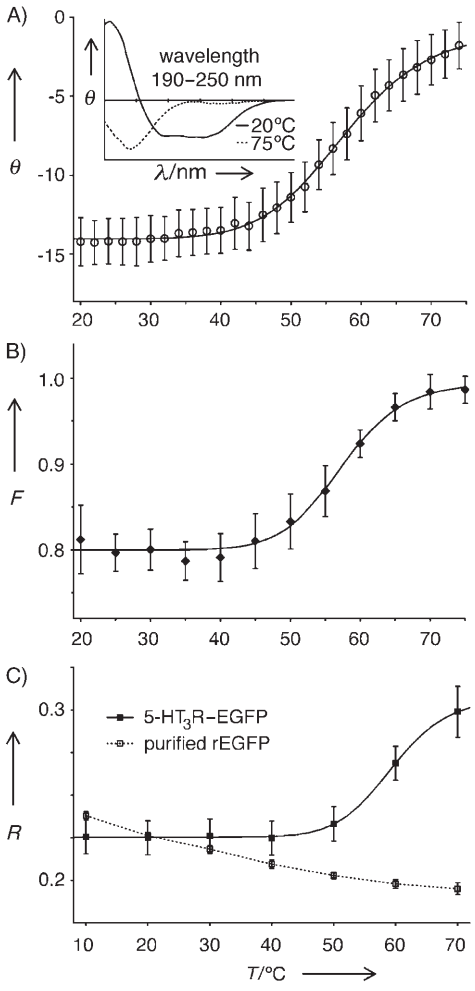

Figure 2. Thermotropic conformational changes of 5-HT_3R. A) Temperature dependence of the molar ellipticity (θ) at 222 nm of detergent solubilized unlabeled 5-HT_3R. Inset shows CD spectra of the receptor recorded at 20 and 75 °C, respectively. B) Temperature dependence of the fluorescence intensity (F) of detergent-solubilized 5-HT_3R–EGFP receptors. Values are normalized to the maximum intensity. C) Temperature dependence of the fluorescence anisotropy (R) of detergent-solubilized 5-HT_3R–EGFP receptors (■) and of purified rEGFP (□); excitation was at 480 nm; fluorescence was measured at 512 nm.

of the five EGFP molecules in the assembled pentameric receptor cause specific fluorescence properties that have been shown here to be sensitive to changes of the receptor structure.

Homo-FRET senses channel activation

Next we considered the possibility of monitoring conformational changes upon receptor activation directly by fluorescence spectroscopy. To assess structural changes by fluorescence-intensity measurements, solubilized 5-HT_3R–EGFP was exposed to different concentrations of the specific receptor agonist 1-(*m*-chlorophenyl)biguanide (*m*CPBG).[27] After 3 min of incubation, the EGFP-fluorescence intensity revealed an agonist dose–response relationship that reached a plateau at saturating concentrations (Figure 3). The calculated EC_{50} of 0.5 ± 0.2 μM was consistent with electrophysiology data obtained with HEK293 cells that express the EGFP-tagged 5-HT_3R ($EC_{50} = 0.66 \pm 0.08$ μM). This suggests that not only thermal but also agonist-induced changes in the receptor conformation can be detected by changes in the intrinsic EGFP-fluorescence intensity (Table 1). Although the ligand-induced fluorescence changes were smaller than those brought about by complete thermal denaturation, they indicated distinct conformational changes in the receptor. When the solubilized receptor was preincubated with an excess of the competing antagonist granisetron (1 μM), the subsequent addition of *m*CPBG did not lead to an increase in EGFP fluorescence intensity. For comparison, we also measured 5-HT_3R–EGFP fluorescence in the presence of a saturating concentration of serotonin (100 μM), the natural agonist of 5-HT_3R. Interestingly, the normalized fluorescence intensity at this treatment was $I/I_0 = 1.2 \pm 0.02$, which was again suppressed in the presence of granisetron (1 μM). Moreover, the nonfluorescent antagonist granisetron alone did not have any effect on the EGFP emission intensity. This demonstrates the high specificity of our measurements and confirms the observed effect of agonist binding on receptor conformation.

The changes in EGFP fluorescence intensity that we observed after agonist application were stable over several minutes and most likely reflect the transition to the desensitized state of the receptor. Support for this hypothesis comes from studies on the structurally related acetylcholine receptor, which show that the majority of the receptor molecules adopt a desensitized structure in the presence of an agonist.[28,29]

Substituted cystein-accessibility studies have indicated that the second transmembrane domain of 5-HT_3R moves during channel activation.[30] Also, in the structurally related $GABA_A$ and acetylcholine receptors channel activation seems to be correlated with the rotation of the second transmembrane domain of the receptor subunits.[31–34] Here we provide the first evidence of agonist-mediated structural changes in the large intracellular loop of 5-HT_3R, which is rather distant from the central channel gate. This suggests possible conformational changes in the third and fourth transmembrane domains. Evidence for structural changes at the interface between the M4 transmembrane region and extracellular ligand-binding domain upon receptor activation were recently provided by studies with the α subunit of the nicotinic acetylcholine receptor.[15]

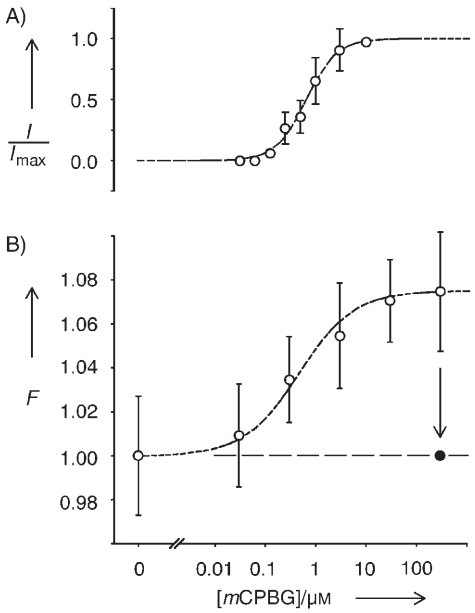

Figure 3. Influence of *m*CPBG on the ion-channel activity and intrinsic fluorescence of 5-HT_3R–EGFP. A) 5-HT_3R–EGFP was transiently expressed in HEK293 cells. Patch-clamp measurements at a holding potential of −60 mV in whole-cell configuration were performed 48 h after transfection, according to a protocol described in detail elsewhere.[18] Peak currents (*I*) are shown at various concentrations of *m*CPBG that were normalized to the maximal peak current, I_{max}, which was achieved at saturating agonist concentration (10 μM *m*CPBG). Each data point represents the mean ± standard error (SE) from measurements on three different cells. Full-line segments represent fit (by using Equation (1) from ref. [18]) to experimental data and yield a half maximal effective concentration (EC_{50}) of 0.66 ± 0.08 μM and a Hill coefficient of $n = 1.4 \pm 0.2$. B) Plasma membranes isolated from HEK293 cells that transiently expressed 5HT_3R–EGFP were solubilized in buffer 2 (see Experimental Section). The EGFP fluorescence of detergent-solubilized 5-HT_3R–EGFP was measured (excitation = 488 nm, emission = 512 nm) and plotted as normalized intensity, $F = I/I_0$ (where I_0 is the fluorescence intensity of 5-HT_3R–EGFP in the absence of *m*CPBG), as a function of the corresponding concentrations of *m*CPBG. Each point (○) represents the mean ± SE of measurements obtained from three independent receptor preparations. The SE of the normalized fluorescence intensities obtained with one series of measurements from one particular receptor preparation was only ±0.01. The normalized-fluorescence intensity of 5-HT_3R–EGFP with *m*CPBG (300 μM) in the presence of the competing antagonist granisetron (1 μM) is also shown (●). This is identical to the fluorescence intensity of the receptor in the absence of any ligand.

We have demonstrated that agonist-induced conformational changes can be sensed by homo-FRET between neighboring EGFP molecules that are inserted in the receptor structure. Our approach has implications for novel functional screening of ligand-gated ion channels by various fluorescence techniques.[39] The optical readout of our assay allows discrimination between agonists and antagonists and has the potential to be used in high-throughput applications. Furthermore, time-resolved measurements might distinguish structural changes evoked by ligand binding from those of channel gating and desensitization. Such distinctions could also be of importance for drug screening.

Experimental Section

Materials: Materials were purchased from the following suppliers: synthetic oligonucleotides (MWG-Biotech AG, Ebersberg, Germany); kits for plasmid and DNA-fragment purification (QIAGEN GmbH, Hilden, Germany); restriction endonucleases (New England Biolabs, MA, USA); purified rEGFP (Clontech, CA, USA); 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-[^{3}H]methyl-1*H*-indol-3-yl)propanone ([^{3}H]-GR65630; 85.5 Ci mmol^{-1}; NEN-DuPont, Boston, MA, USA); quipazine and 1-(*m*-chlorophenyl)biguanide (*m*CPBG; Tocris-Cookson, Langford, UK); granisetron (Apin Chemicals, Abingdon, UK).

DNA constructs: The 5-HT$_3$R constructs were obtained from a plasmid that contained the short-spliced variant of the murine 5-HT$_{3A}$R, which corresponds to the SwissProt entry p23979. The construct was under the transcriptional control of the cytomegalovirus (CMV) promoter.[35] In order to purify the protein, a hexa-histidine tag was fused to the N terminus of 5-HT$_3$R by site-directed mutagenesis (Quickchange Kit, Stratagene, CA, USA) with the oligonucleotides 5′-GCC GGA GGA GGG CCA CTA GTC ATC ACC ATC ACC ATC ACC AGG AGG ATA CCA CCC-3′ and 5′-GGG TGG TAT CCT CCT GGT GAT GGT GAT GGT GAT GAC TAG TGG CCC TCC TCC GGC-3′. This 5HT$_3$R-N–His construct was confirmed by DNA sequencing. The 5HT$_3$R–EGFP fusion protein was obtained following a previously described protocol.[18]

Cell culture and transfections: Adherent human embryonic kidney cells, HEK293, were grown as described elsewhere.[35] For electrophysiology, confocal microscopy, and membrane purification cells were seeded at a density of 150 000 cells mL^{-1} either in cell-culture dishes (35 mm), six-well plates that contained glass cover slips (22 mm diameter), or flasks (150 cm^2). The cells were transfected by using Effectene reagent (Qiagen GmbH, Hilden, Germany). Experiments were performed 48 h after transfection.

Membrane solubilization: All manipulations were performed on ice or at 4 °C. Cell pellets (4 g, wet weight) were resuspended in buffer 1 (10 mL; 10 mM HEPES, 1 mM EDTA, pH 7.4) and homogenized for 90 s with an Ultra-Turrax T25 (IKA, Staufen, Germany). Membrane fractions were collected by centrifugation at 27 000 *g* for 40 min. Membrane proteins were solubilized in buffer 2 (20 mL; 50 mM NaPi, 300 mM NaCl, 50 cmc (critical micelle concentration) $C_{12}E_9$, pH 8.0). A final centrifugation at 100 000 *g* for 60 min removed the remaining membrane fractions. The supernatant was collected and stored at −80 °C.

Receptor purification: Nickel nitrotriacetic acid agarose (5 mL; Qiagen, Hilden, Germany) was washed three times with buffer 2, incubated at 4 °C overnight with solubilized 5-HT$_3$R-N–His, and packed into a column. Nonspecifically bound protein was removed by increased concentrations of imidazole in NaPi (10 mM), NaCl (500 mM), $C_{12}E_9$ (25 cmc), pH 7.4. Protein elution was followed by absorbance measurements at 280 nm. The receptor was collected after application of imidazole (250 mM). The purified receptor was desalted by gel filtration on a G-25 column (NAP-l0, Pharmacia Biotech, Uppsala, Sweden) by using equilibration and elution buffer 3 (1 mM Tris, 5 cmc $C_{12}E_9$, pH 7.4).

Receptor characterization by electrophysiology and radioligand binding: Standard whole cell patch-clamp measurements were performed as described previously.[18] The affinity of 5-HT$_3$R (mutant) proteins for radioligands and the total amount of ligand-binding sites were determined as described elsewhere.[35,36]

CD measurements: CD spectra of purified 5-HT$_3$R-N–His receptors were obtained on an Aviv 62DS spectrometer (Aviv, Lakewood, NJ, USA) with the protein (10 nM) in $C_{12}E_9$ (5 cmc). Temperature-dependence of the molar ellipticity at 222 nm was measured at a heating rate of 1 °C min^{-1}. The protein secondary structure was evaluated from the CD spectra as described elsewhere.[37,38]

Fluorescence measurements: were performed on a SPEX Fluorolog II (Instruments S.A., Stanmore, UK) by using 1.5 nm excitation and emission bandwidths. Purified rEGFP was dissolved in buffer 2. Sample solutions in quartz cuvettes (1 × 1 cm^2; Hellma, Müllheim, Germany) were placed in a temperature-controlled holder and were continuously stirred. For anisotropy measurements, Glan Taylor polarizers (Halbo Optics, Chelmsford, UK) were placed in the excitation and emission light paths.

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