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Abstract
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mKikGR, a Monomeric Photoswitchable Fluorescent Protein

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Abstract

The recent demonstration and utilization of fluorescent proteins whose fluorescence can be switched on and off has greatly expanded the toolkit of molecular and cell biology. These photoswitchable proteins have facilitated the characterization of specifically tagged molecular species in the cell and have enabled fluorescence imaging of intracellular structures with a resolution far below the classical diffraction limit of light. Applications are limited, however, by the fast photobleaching, slow photoswitching, and oligomerization typical for photoswitchable proteins currently available. Here, we report the molecular cloning and spectroscopic characterization of mKikGR, a monomeric version of the previously reported KikGR that displays high photostability and switching rates. Furthermore, we present single-molecule imaging experiments that demonstrate that individual mKikGR proteins can be localized with a precision of better than 10 nanometers, suggesting their suitability for super-resolution imaging.

Introduction

Green fluorescent proteins (GFPs) and GFP-like fluorescent proteins have found extensive use in molecular and cellular biology [1–4]. Recently, photoswitchable fluorescent proteins have been reported [5–13] whose fluorescence properties can be altered upon illumination at specific wavelengths. The controlled photoconversion of these proteins provides unique opportunities to mark and track selected molecules in cells and organelles [14,15]. To gain access to molecular dynamics on short timescales, two important requirements are an efficient and rapid photoswitching into the ‘on’ state, and a bright fluorescence after switching.

Another promising application of photoswitchable proteins is their use in super-resolution microscopy. This technique relies on the stochastic photoactivation and localization of single molecules (PALM, STORM, FPALM), in which a fluorescence image is constructed from the high-accuracy localization of individual fluorescent molecules that are switched on and off optically [16–20]. Since the localization accuracy is determined by the total photon number detected from individual molecules, the brightness and photostability of the photoswitchable molecules represent the key parameters to achieve high spatial resolution [21].

KikGR, a mutant of a fluorescent protein cloned from the stony coral Favia favus, emits bright green fluorescence in its initial state, but is switched into a red emitting species upon illumination with UV or violet light [11]. Since the photoswitching of KikGR is efficient (φw = 4.7 × 10−3) and the red emitting KikGR shows a bright fluorescence (φR = 0.65), this protein could be an excellent fluorescent tag for both selective cell labeling and photoactivation localization microscopy. However, KikGR needs to form a homotetrameric complex to become fluorescent similar to other GFP-like proteins [5,22]. The necessity for a noncovalent oligomerization limits its use in applications with protein fusions.

Here we report a monomeric version of KikGR, mKikGR. We characterize the spectroscopic properties of mKikGR, and show that the protein retains the advantageous spectroscopic properties of KikGR including efficient switching and bright fluorescence.

Results and Discussion

Construction of mKikGR

We performed a site-directed random mutagenesis approach on KikGR. The products were also subjected to error-prone PCR to introduce additional variation at other positions that might affect the photoconversion reaction or protein folding. E. coli cells transformed with plasmids carrying the mutated DNA were plated and screened for photoswitching behavior using a home-made image analyzing system. Then, protein samples were subjected to pseudo-native PAGE analysis to examine their oligomeric state. The directed evolution of KikGR towards a monomeric version was achieved after 15 cycles of mutagenesis. The introduction of 21 mutations, some of which are located in the tetramer interfaces, transformed KikGR into a monomeric version, mKikGR (Figure 1A). We determined the absolute molecular mass of mKikGR to be 30.0 kDa by analytical equilibrium ultracentrifugation analysis. The similarity of this
value to that deduced from the primary structure of the protein, 26.5 kDa, confirms its monomeric nature. A mammalian expression plasmid was generated that encodes a chimeric protein comprising mKikGR and human β-actin. Twelve hours post-transfection into primary cultured astrocytes, stress fibers were clearly visible and locally highlighted (Figure 1B).

Spectroscopic characterization

The absorption spectrum of mKikGR displays two distinct maxima, at 505 nm (ε = 4.9×10^4 M^-1 cm^-1) and 390 nm (ε = 1.2×10^4 M^-1 cm^-1) (Figure 2A, green solid line). The pH dependence of these two absorptions (Figure 2B) demonstrates that the 505-nm band corresponds to the deprotonated form of the mKikGR chromophore, and the band at 390 nm corresponds to the protonated form. While the protonated form of the chromophore was nearly non-fluorescent (data not shown), the deprotonated form of the chromophore (green mKikGR) was converted into a red chromophore. The red mKikGR displayed two bands at 591 nm (ε = 1.8×10^4 M^-1 cm^-1) and 359 nm (ε = 1.2×10^4 M^-1 cm^-1) (Figure 2A, red solid line). The pH dependence of these two absorptions (Figure 2C) suggests that both bands are together with pH dependence of the absorption spectrum of the mKikGR chromophore at pH 7.0 upon illumination at 405 nm (12 mW cm^-2). The absorption band of the green mKikGR was reduced and concomitantly the absorption band of the red mKikGR increased. The spectra display isosbestic points at 519 and 371 nm, indicating that the switching of the chromophore can be described as a simple conversion between two states. Time evolutions of the peak absorption of the red mKikGR can be fitted by a first-order kinetic model (Figure 3A inset), giving a rate of photoswitching of 3.1×10^-3 s^-1. SDS/PAGE experiments demonstrated fragmentation of mKikGR upon illumination. While the green mKikGR showed a single band at 28 kDa, the red mKikGR showed two bands at 18 and 10 kDa (data not shown). This result strongly suggests that the β-elimination reaction observed in the photoactivatable protein Kaede also occurs in the photoswitching of mKikGR [5,23,24].

The rate of photoswitching depends significantly on pH. The switching rate at pH 5.0 (ksw_405 nm = 6.8×10^-3 s^-1) is about ten-fold faster than that at pH 8.0 (ksw_405 nm = 6.7×10^-4 s^-1) (Fig. 3B). Furthermore, the photoswitching rate is nearly proportional to the absorption of the protonated form at 405 nm (Figure 3E). These results suggest that the photoswitching reaction initiates from the protonated state of the green mKikGR [5,10]. The quantum yield of the photoswitching (Φsw) is 7.5×10^-3, as calculated from the absorption cross-section of mKikGR at 405 nm (3.83×10^-17 cm^2), the rate of the photoswitching, and the illumination intensity of the 405 nm light (11.6 mW cm^-2). This value is somewhat larger than that of kikGR (Φsw = 4.7×10^-5) [11]. The rate of the photoswitching at a constant pH increases linearly with the illumination power of the 405 nm light (Figure 3C), suggesting that the photoswitching reaction occurs through a one-photon excitation mechanism. The reaction scheme of the photoswitching is drawn in Figure 4 [24,25].

Figure 3D and 3E show fluorescence spectra of mKikGR excited at 475 nm (Figure 3D) and 553 nm (Figure 3E) before (broken lines) and after photoswitching (solid lines) by illuminating at 405 nm. The photoswitching resulted in a 560-fold increase in the ratio of red to green fluorescence (R/G), from 0.043 to 24.0. Figure 3F shows fluorescence images of mKikGR (10 μM) embedded in a polyacrylamide gel. After a single pulse (1 sec)

Figure 1. Construction of mKikGR. (A) Amino-acid sequences for KikG, KikGR, and mKikGR. (B) Rat primary astrocyte expressing mKikGR-actin. Images show before (left) and after (right) local photoswitching by irradiating 405 nm laser over the yellow-boxed region. Cells are cultured at 37°C. doi:10.1371/journal.pone.0003944.g001
Figure 2. Spectroscopic properties of the green and red form of mKikGR. (A) Normalized absorption (solid line) and fluorescence (dashed line) spectra of the green mKikGR (4.7 μM, green line) and red mKikGR (4.7 μM, red line). The red mKikGR was obtained by illuminating at 405 nm (12 mW cm⁻²) for 90 minutes. Fluorescence spectra of the green and red mKikGR were measured with 475 nm and 555 nm excitation, respectively. All measurements were performed at pH 8.0. (B, D) pH dependence of absorption spectra of the green (B) and red (D) mKikGR (4.7 μM). (C, E) pH dependence of fluorescence spectra of the green (C) and red (E) mKikGR (4.7 μM). Fluorescence spectra of the green and red mKikGR were measured with 475 nm and 555 nm excitation, respectively. (Inset) Peak fluorescence intensities at different pH. The solid lines show fitting with the Henderson-Hasselbalch equation.

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illumination with 405 nm light (6.3 W cm^{-2}), a significant decrease of the green fluorescence (bottom left) and increase of the red fluorescence (bottom right) were observed. The R/G ratio decreased of the green fluorescence (bottom left) and increase of the red fluorescence (bottom right) were observed. The R/G ratio were observed. The R/G ratio.

Photobleaching of red mKikGR

Table 1. Spectroscopic properties of monomeric photoswitchable proteins which change color on illumination.

| Protein | \( \Delta \varphi_{sw} \) | \( \varphi_{in}^{sw} \) | \( \varepsilon^{sw} \) | \( \varphi_{sw}^{sw} \) | \( \varepsilon^{sw} \) | \( \varphi_{bl} \) |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| PS-CFP  | N.A.            | 0.34 \times 10^{-4} | 0.19            | 2.7 \times 10^{-4} | N.A.            |                |
| mEosFP  | N.A.            | 0.07             | 0.34 \times 10^{-4} | 0.62            | 3.7 \times 10^{-4} | 3.0 \times 10^{-5} |
| Dendra  | N.A.            | 0.72             | 2.1 \times 10^{-4} | 0.70            | 2.0 \times 10^{-4} | N.A.            |
| mKikGR  | 7.5 \times 10^{-3} | 0.69            | 4.9 \times 10^{-4} | 0.93            | 2.8 \times 10^{-4} | 6.5 \times 10^{-4} |
| KikGR   | 4.7 \times 10^{-3} | 0.70            | 2.1 \times 10^{-4} | 0.65            | 3.3 \times 10^{-4} | N.A.            |

\( \Delta \varphi_{sw} \): quantum yield of photoswitching, \( \varphi_{in}^{sw} \): fluorescence quantum yield of an initial state, \( \epsilon^{sw} \): molar extinction coefficient of an initial state, \( \varphi_{sw}^{sw} \): fluorescence quantum yield of a photoswitched state, \( \epsilon^{sw} \): molar extinction coefficient of a photoswitched state, \( \varphi_{bl} \): photobleaching quantum yield of the photoswitched state.

The spectroscopic properties of KikGR are also listed as a reference.

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Superresolution position determination of individual mKikGR proteins

The monomeric mKikGR displays bright fluorescence (\( \varphi_{in}^{sw} = 0.63 \)), a large quantum yield of photoswitching (\( \varphi_{in}^{sw} = 7.5 \times 10^{-5} \)), and a low quantum yield of photobleaching (\( \varphi_{bl} = 6.47 \times 10^{-6} \)). Those spectroscopic properties of mKikGR make the protein a candidate for photoactivation localization microscopy (PALM) [16]. The principle of PALM relies on the stochastic activation of spatially well-separated fluorescent proteins and the ability to determine their location with a precision much better than the diffraction limit. When after localization the proteins are photobleached and new ones nearby are activated, their relative positions are obtained and a local structure can be reconstructed [16–20]. The resolution of the resultant reconstructed image is ultimately determined by the accuracy of position determination of the individual molecules, a value that is limited by the number of photons that can be emitted by a single molecule before photobleaching.

The significantly improved photobleaching stability of mKikGR compared to other monomeric photoactivatable proteins suggests that use of mKikGR in localization microscopy could result in a further improvement of resolution. Here we determined localization accuracies obtained from individual mKikGR molecules. Figure 7A shows a typical fluorescence intensity trajectory of a single mKikGR in the red state, recorded every second with 100 ms integration time (excited at 568 nm). The molecule was photoswitched from the green to red at \( t = 41 \) sec. The trajectory showed large intensity fluctuations, which corresponds to blinking and is typical for fluorescent proteins [20]. We fitted the single-molecule fluorescence spot with a 2D Gaussian profile, determined its centroid, and retracted this second until photobleaching occurred. Figure 7B shows the distribution of the centroid positions determined from the time series of images of the single mKikGR protein whose intensity trace is shown in Figure 7A. The standard deviation of the set of positions is 8.8 nm and represents the localization accuracy obtained from a single 100-ms acquisition.

Figure 7C (solid circles) shows the relationship between the localization precision calculated from the sets of centroid positions derived from a single mKikGR and the total number of photons present in a single-molecule image. We also analyzed individual qdot molecules (qplot655) as a reference (Figure 7C, open circles). The solid line in Figure 7C is a theoretical localization precision calculated by the equation [21],

\[
\sigma^2 = \frac{1}{N} + \frac{q^2}{12N} + \frac{8\pi\rho^2R^2}{q^2N^2}
\]

where \( \sigma \) is the standard deviation of the point spread function (150 nm), \( N \) is the total number of photons collected, \( q \) is the size of.
Figure 3. Kinetics of photoswitching from the green to red mKikGR. (A) Time course of the absorption spectra of mKikGR (4.7 μM, pH 7.5) on illumination at 405 nm (12 mW cm⁻²). (Inset) Time course of the peak absorbance of the red mKikGR (580 nm). The solid line shows the fitting with a first-order kinetic model. (B) pH dependence of the rate of the photoswitching (solid circles, left axis) and absorbance at 405 nm (open circles, right axis). (C) Excitation power dependence of the photoswitching. The rates were determined at pH 5.0. (D, E) Fluorescence spectra of mKikGR before (dashed lines) and after (solid lines) photoswitching. The spectra were measured with 475 nm (D) excitation and 555 nm (E) excitation. (F) The top panels show fluorescence images of the green (left) and red (right) mKikGR embedded in the thin film of polyacrylamide gel (10 μM, pH 7.0). The wavelength regions that emission filters transmit are indicated by shadow in Fig. 2D (green) and 2E (red). The region indicated by circle was illuminated with 405 nm light (1 sec, 6.3 W cm⁻²). The bottom panels show fluorescence images of the green (left) and red (right) mKikGR.

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an image pixel (166 nm), and $b$ is the background noise per pixel (0.5 photon per pixel). The theoretically calculated localization precision agrees well with the experimental data. The localization precision obtained from mKikGR molecules are about 40% less than that of the reference sample. This is partially due to the less bright fluorescence of mKikGR as compared with the reference sample which results in a higher background signal. The blinking behavior of mKikGR (see Figure 7A) also affects the signal to noise ratio which limits the localization precision.

Our results demonstrate that mKikGR is suitable for localization microscopy using fluorescent proteins. The small photobleaching yield ($6.5 \times 10^{-6}$) is an advantage of mKikGR over reversible photoswitchable fluorescence proteins such as Dronpa which shows a photoswitching yield (from the bright to the dark state) of $3.2 \times 10^{-4}$. Although it was demonstrated that the use of Dronpa is advantageous in photoactivation localization microscopy [20,29] due to its efficient switching from the bright to the dark state [8,30,31], this rapid switching results in lower number of photons collected in single images that result in a limited spatial resolution. The large number of photons obtained from red mKikGR is useful not only in photoactivation localization microscopy, but also for single-molecule tracking. A localization precision of about 30 nm can be achieved with 100 collected photons (see Figure 7C). This result together with the total number of photons that can be collected from a single red mKikGR suggest that one should be able to record 100 images with 30 nm localization precision, which meets the requirements in most of single-molecule tracking experiments.

We have produced a highly photostable, monomeric photoactivatable fluorescent protein, and characterized its photophysical properties. Its monomeric form allows it to be used as a highly specific and stable fluorescent tag. We have demonstrated that its increased photostability can be used to improve resolution of position determination, illustrating its applicability to superresolution localization microscopy. An important future direction will be the expansion of the arsenal of photoswitchable proteins to different emission wavelengths allowing for multicolor imaging.[5]

**Materials and Methods**

**Mutagenesis**

Site-directed semi-random mutations were introduced as described previously [32]. Multiple degenerative primers, greater than ten in some cases, were used together for reactions. Additional random mutations were introduced using the error-prone PCR [33].

**Analytical ultracentrifugation**

Sedimentation equilibrium experiments were performed using a Beckman X1-1 analytical ultracentrifuge at 20°C. Absorbance was measured at the maximum wavelength as a function of radius at 25,000 rpm.

**Protein expression, purification, pH titration, SDS/PAGE**

Recombinant mKikGR protein was expressed in *E. coli*. The protein was purified with Ni-NTA chromatography (Qiagen) [5,34], and dialyzed into 50 mM Tris at pH 8.0, 300 mM NaCl, 1 mM DTT, and 10% Glycerol. The solutions for pH titration contained 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 25 mM of one of the buffers – acetate, Mes, Hepes, Tris, or bicarbonate. 1 N NaOH and 1 N HCl solutions were used to obtain the correct pH. SDS/PAGE analysis was done with 12% polyacrylamide gels (Bio-Rad).

**Ensemble spectroscopy**

Absorption spectra were measured with a spectrophotometer (PerkinElmer) using a UV-transparent cuvette (Brandtech). For calculation of molar extinction coefficients, protein concentrations...
Figure 5. Photoswitching of mKikGR at the single-molecule level. (A) Fluorescence images of individual red mKikGR molecules embedded in polyacrylamide gel (100 pM, pH 7.0). The images were recorded with 568 nm excitation (130 W cm$^{-2}$, 100 ms integration, 1 Hz). The sample was illuminated with 405 nm light (33 mW cm$^{-2}$) between 568 nm pulses. The images were recorded after 0, 1.8, 8.1, and 22 seconds total illumination time with 405 nm light. (B) Time course of integrated intensities of the images. The solid line shows fitting with a first order kinetic model. (C) Excitation power dependence of the photoswitching rates determined from the single-molecule measurements. The solid line shows theoretical switching rate calculated from bulk experiments.
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Figure 6. Single-molecule photobleaching of mKikGR. (A) Schematic illustration of experimental configuration. The excitation power of the 405 nm and 568 nm lights were set at 6.3 and 130 W cm$^{-2}$, respectively. (B) Time course of the fluorescence intensity of the red mKikGR embedded in polyacrylamide gel (100 pM, pH 7.0). The red and blue lines show a single- and double-exponential fit. (C) Excitation power dependence of the photobleaching rates of the red mKikGR.
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were determined using a Bradford assay kit (Bio-Rad) with BSA as a standard. Fluorescence spectra were recorded with a fluorimeter (Photon Technology International) using a quartz cuvette. The fluorescence quantum yields of the green and red mKikGR were determined using fluorescein ($\phi_f = 0.95$ in 0.1 M NaOH) and sulforhodamine 101 ($\phi_f = 0.98$ in ethanol) as references. We photoswitched mKikGR (500 µM, $5 \times 10^{-6}$ M) by illuminating the samples with a diode-pumped solid-state laser at 405 nm (CrystaLaser) at a power of 1.2–12 mW cm$^{-2}$.

Single-molecule imaging

10 µl of a solution containing 100 pM mKikGR and 15% polyacrylamide was deposited on a clean coverslip. A second coverslip was placed on top of the sample to produce a thin film of mKikGR-containing gel. The samples were mounted on an inverted microscope (IX-71, Olympus). 568-nm light from an Ar-Kr ion laser (Innova 70C-Spectrum, Coherent Inc.) and 405-nm light was focused into the back-focal plane of the microscope objective (PlanApo, N.A. = 1.45, Olympus) to create a collimated beam, resulting in the illumination of a circular area with a diameter of 50 µm at the sample plane. The fluorescence of the samples was collected by the same objective and, after passing a dichroic mirror (T585lp) and emission filter (ET620/60), focused onto an EM-CCD camera (iXon, Andor Technology). The images were further magnified 1.6 times with a lens before the camera, resulting in a pixel size of 166 nm x 166 nm. While the fluorescence images of the red mKikGR molecules were recorded with the 568 nm excitation light (100 msec integration, 1 Hz), the 405 nm light was introduced into the microscope between the 568 nm pulses to photoswitch mKikGR molecules from the green to red form. The illumination timing was controlled by shutters (Uniblitz).

A quantum dot sample was prepared by depositing 1 pM Qdot655 (Molecular Probes) in TE buffer (20 mM Tris at pH 7.5, 2 mM EDTA) on a clean coverslip. The images were recorded in a similar way as for the mKikGR sample. The 488-nm line of the Ar-Kr laser was used as an excitation source.

The fluorescence images were analyzed using MATLAB script designed and written in house for particle detection and localization.

Author Contributions

Conceived and designed the experiments: SH AM AMvO. Performed the experiments: SH HT ABK. Analyzed the data: SH HT. Contributed reagents/materials/analysis tools: HT ABK AM. Wrote the paper: SH AM AMvO.
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