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Insight into the structure and mechanism of the reversible photoswitch of a fluorescent protein

A multi-departmental research approach

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Structure and mechanism of a molecular light switch

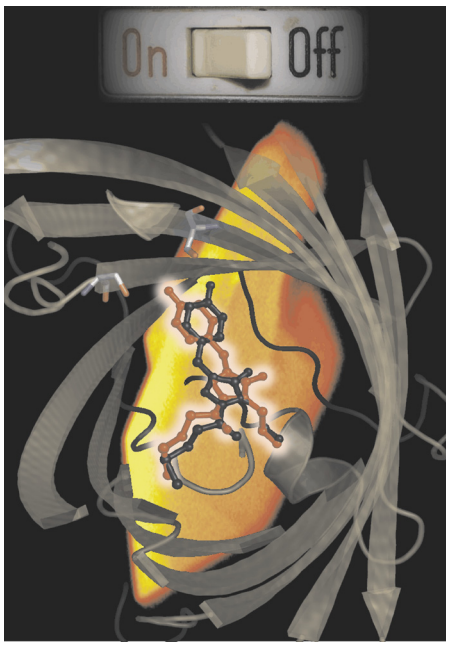
Fluorescent proteins, like the well known green fluorescent protein (GFP) are widely used to generate fusion tags that act as fluorescent reporters for studying protein localizations and dynamics in live cells. Recently, with asFP595 isolated from the sea anemone *Anemonia sulcata* (Fig. 1) and Dronpa cloned from the coral Pectiniidae, the first two members of the new class of reversibly switchable fluorescent proteins (RSFP) have been described. RSFPs can be transferred by light irradiation from a non-fluorescent 'off' to a fluorescent 'on' state and back again.

Somewhat related fluorescent proteins, namely photoactivatable proteins like PA-GFP or PS-CFP, that can be irreversibly switched by light from one state into a different state have also stirred a lot of interest in recent years. However, photoactivatable proteins can be switched only once and not back to their initial state.

Hence the switching reversibility of RSFPs is a very unique feature that may allow fundamentally new applications.

Like other fluorescent proteins, RSFPs may be fused by genetic methods to a host protein. However, whereas the 'classical' fluorescent proteins are practically always in their on-state, until they are irreversibly bleached, the switchability of asFP595 and Dronpa may provide the tools to get new insights into the diffusive or directed movement of the host proteins. The intriguing features of the RSFPs make many other applications imaginable. For example, the reversible photoswitching of fluorescent markers may provide nanoscale resolution in fluorescence microscopy using lenses and regular illumination.

The RSFP asFP595, used in our studies, has some favorable switching properties: (i) Switching from a non-fluorescent off-state to a fluorescent on-state is induced by irradiation with green light. (ii) Red fluorescence emission of the on-state is elicited by the same green light. And (iii) the asFP595 chromophore in the on-state reverts eventually back to the off-state, but this transition can also be promptly induced by gentle irradiation with blue light. In other words, green light is required to switch on and to excite; blue light is used to switch off the fluorophore. Currently, however, despite its promising properties, the wild type asFP595 protein, as isolated



Zusammenfassung

Schaltbare Proteine, die sich durch Bestrahlung mit sichtbarem Licht reversibel zwischen einem fluoreszierenden ‚Ein'- und einem nicht-fluoreszierenden ‚Aus'-Zustand hin und herschalten lassen, sind erst seit wenigen Jahren bekannt. Derzeit

Fortsetzung auf Seite 3

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Fig. 1. Snakelocks anemone (*Anemonia sulcata*). Photography: Richard Lockett.

Abb. 1: Wachsrose (*Anemonia sulcata*). Bild: Richard Lockett.

from the Snakelocks anemone, is far from being an optimal tag. Therefore optimization of this tag is needed. To this end, researchers from three departments of this institute set out in a collaborative effort to understand the structure and the switching mechanism of asFP595.

Structure of asFP595

First, we determined the atomic off-state structure of asFP595. Large amounts of the protein were produced in bacteria, purified and crystallized. After solving the x-ray structure, we (and independently two other groups) discovered that its structure is very similar to that of GFP. Like many other fluorescent proteins, asFP595 forms tetramers. It is a globular protein, with a very rigid structure, so that the chromophoric region is shielded from the environment. The chromophore resides in a helical segment that is enclosed by a β-barrel (Fig. 2). The chromophore is formed by three amino acids (M63-Y64-G65) that rearrange to a conjugated system with a p-hydroxyphenyl and an imidazolinone ring that are connected by a methine bridge. Remarkably, in asFP595, the protein backbone is broken at the chromophore. This backbone break is unique for a fluorescent protein and it appears that this break provides the protein with the intrinsic flexibility to allow switching of the chromophore with low light energies.

The on- and the off-state

In order to understand the structural differences between the on- and the off-state, we used the protein variant asFP595-A143S, which exhibits slower switching kinetics than the wild type protein. We found that asFP595-A143S protein crystals can be reversibly transferred by light between the on- and the off-state. Non-irradiated asFP595-A143S crystals are barely fluorescent, whereas after irradiation with green light, they display bright red fluorescence that can be switched off with blue light (Fig. 3). Again, this switching is reversible numerous times. The switching kinetics of the protein in solution is very similar to the kinetics of the protein within the crystal lattice. The crystallized proteins thus behave as in solution. Therefore crystals were irradiated for 0, 1, or 5 minutes with green light and subsequently captured by flash freezing in liquid nitrogen to obtain structures of the protein in its off- and on-states.

We found that the on- and the off-state chromophores adopt different conformations: The chromophores of the irradiated proteins (on-state) were in a cis-conformation, whereas the those of the non-irradiated proteins (off-state) adopted a trans-conformation (Fig. 4). As expected,

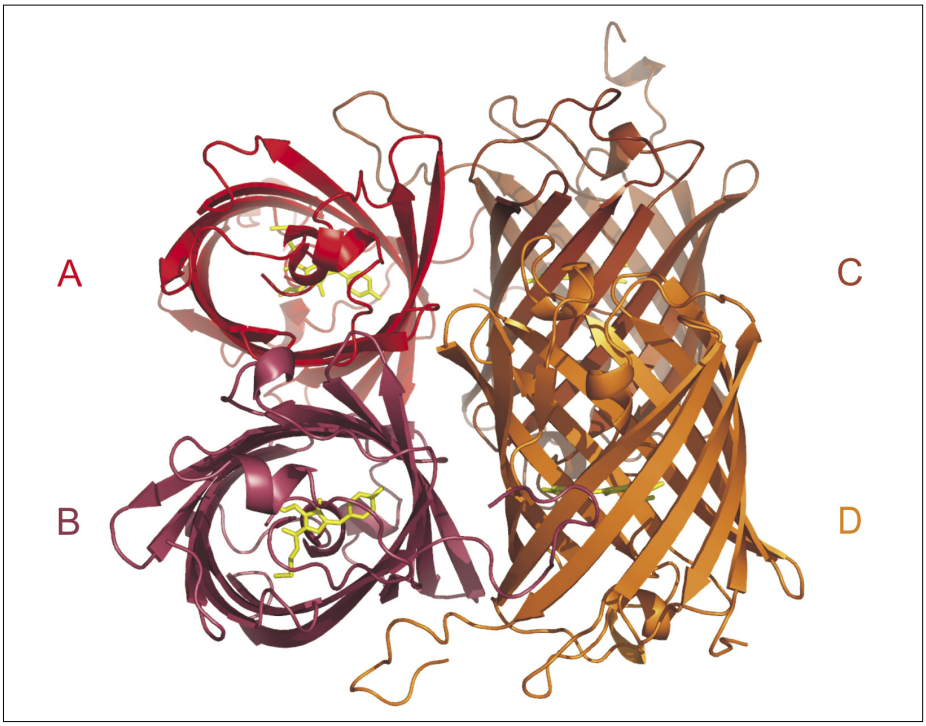

Fig. 2. Overall structure of asFP595. A schematic ribbon representation of the quaternary tetrameric structure of asFP595 showing the four molecules (A-D) in different colors and the chromophores highlighted in yellow.

Abb. 2: Gesamtstruktur von asFP595. In der schematischen Darstellung des tetrameren Proteins sind die vier Moleküle (A-D) in verschieden Farben dargestellt. Die Chromophore sind gelb eingefärbt.

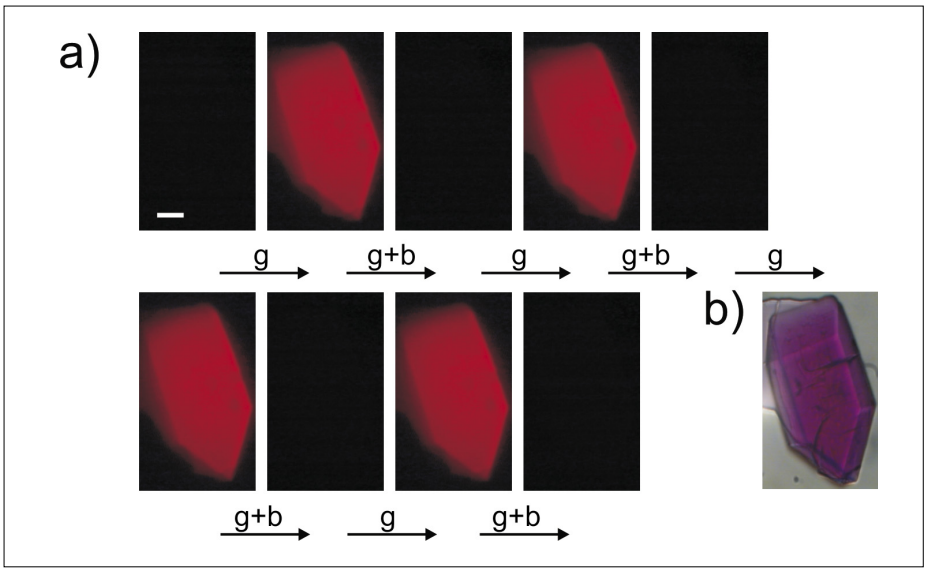

Fig. 3. (a) Reversible photoswitching of an asFP595-A143S protein crystal under a fluorescence microscope. Irradiation with green light (g= 550 ± 20 nm) yields brightly fluorescent crystals. The fluorescence is quenched by gentle irradiation with blue light (b= 450 ± 20 nm). **(b)** Brightfield image of the crystal. Scale bar: 20µm.

Abb. 3: (a) Reversibles Photoschalten von asFP595-A143S Proteinkristallen mit Hilfe eines Fluoreszenzmikroskops. Bestrahlung mit grünem Licht (g= 550 ± 20 nm) ergibt stark fluoreszierende Kristalle. Die Fluoreszenz kann durch schwache Bestrahlung mit blauem Licht (b= 450 ± 20 nm) gelöscht werden. **(b)** Durchlichtbild des Proteinkristalls. Maßstab: 20µm.

in the briefly irradiated crystals, we identified a mixture of chromophores of both conformations.

Spectroscopic data further indicates that in the cis-conformation, the chromophoric p-hydroxyphenyl group is in equilibrium between a (non-fluorescent) protonated and a (fluorescent) non-protonated form. Such a proton transfer would not have been visible in the crystal structure, but it is likely a consequence of the changes in the immediate protein surrounding after a trans-cis-isomerization.

Hence, from this crystallographic analysis, we knew the structural difference between the protein in the on- and the off-state. As a next step, we set out to investigate the molecular movements

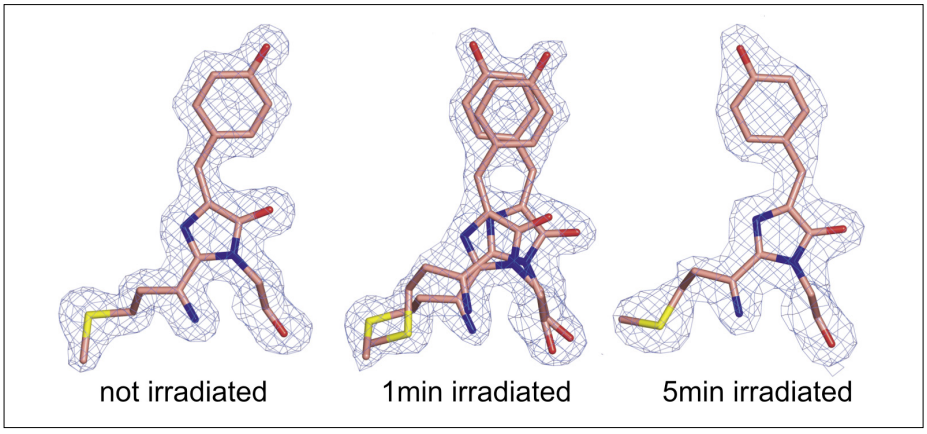

Fig. 4. Trans- and cis-conformation of the MYG chromophore in the 'off'- and the 'on'-state, respectively. Atoms are color-coded by atom type (carbon – salmon, oxygen – red, nitrogen – blue). Final $2F_o$-F_c electron densities around the chromophores are contoured at the 1σ level. For asFP595-A143S irradiated for one minute, both isomerization states are depicted because both structures were observed in a single crystal with equal proportions.

Abb. 4: Trans- und Cis-Konformation des MYG Chromophors im 'Aus'- und im 'An'-Zustand. Die Atome sind farbkodiert dargestellt (Kohlenstoff – lachsfarben, Sauerstoff – rot, Stickstoff – blau). Die $2F_o$-F_c Elektronendichten um die Chromophoren sind auf dem 1σ-Niveau abgebildet. Im Falles des für eine Minute belichteten asFP595-A143S Kristalls sind beide Konformationen dargestellt, da beide Strukturen zu gleichen Anteilen im Kristall bestimmt wurden.

Fortsetzung ...

sind mit den Proteinen asFP595 und Dronpa erst zwei Vertreter solcher reversibel schaltbaren fluoreszierenden Proteine (RSFPs) beschrieben worden. Nichtsdestotrotz verspricht diese neue Proteinklasse aufgrund ihrer einzigartigen Eigenschaften bereits jetzt eine Vielzahl interessanter Anwendungsmöglichkeiten.

Das Protein asFP595, dessen Schaltmechanismus wir in dieser Studie untersucht haben, kommt normalerweise in den Tentakelspitzen der Wachsrose *Anemonia sulcata* vor, einer Seeanemone, die in den lichtdurchfluteten Flachwasserbereichen des Mittelmeers und des Nordatlantiks lebt. asFP595 wird durch Beleuchtung mit grünem Licht von einem nicht-fluoreszierenden ‚Aus'-Zustand in einen fluoreszierenden ‚Ein'-Zustand versetzt. Von diesem ‚Ein'-Zustand fällt es spontan in den ‚Aus'-Zustand zurück, kann aber auch durch Beleuchtung mit blauem Licht zurückgeschaltet werden. Dieses lichtgetriebene Schalten ist reversibel und viele Male wiederholbar. In einer gemeinsamen Studie dreier Abteilungen haben wir die molekulare Struktur von asFP595 bestimmt und wesentliche Teile des Schaltmechanismus aufgeklärt. Diese Untersuchungen haben gezeigt, dass nach der Absorption eines grünen Photons das asFP595-Chromophor von einer Trans- in eine Cis-Position isomerisiert. Molekulardynamik-Rechnungen deuten an, dass das Chromophor bei dieser lichtinduzierten Reaktion eine ‚Hula-Twist'-Bewegung macht. Bei dieser Bewegung dreht sich in erster Linie die Methin-Brücke, welche die beiden aromatischen Ringe des Chromophors verknüpft. Tatsächlich ändert das Chromophor während des Hula-Twists seine Position lediglich um 3×10^{-10} Meter. Diese winzige Änderung reicht aus, um aus dem nicht-fluoreszierenden ein fluoreszierendes Protein zu machen.

Diese neuen Erkenntnisse über die Struktur und den Schaltmechanismus des Proteins sollten es zukünftig ermöglichen, mit Hilfe von gerichteten molekularbiologischen Ansätzen das Protein für weitere Anwendungen zu optimieren. Denkbare Einsatzbereiche eines solchen modifizierten asFP595 reichen von der höchstauflösenden Fluoreszenzmikroskopie bis hin zum Einsatz als Datenspeicher.

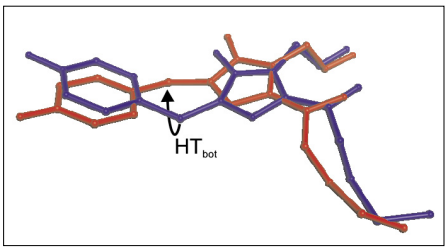

Fig. 5. Hula twist mechanism. Upon irradiation with green light the chromophore changes its conformation from the non-fluorescent trans-conformation (blue) into the fluorescent cis-conformation (red) with a so-called hula twist. In a hula twist mechanism, the two rings of the chromophore almost stay in place and only the methine bridge that connects both rings moves.

Abb. 5: „Hula Twist"-Mechanismus. Bei der lichtinduzierten Konformationsänderung des Chromophors aus der nicht-fluoreszierenden Trans-Form (blau) in die fluoreszierende Cis-Form (rot) dreht sich in erster Linie die Methin-Brücke, welche die beiden aromatischen Ringe des Chromophors verknüpft.

of the chromophore that take place upon irradiation to induce a trans to cis isomerization.

Computer simulations of the switching mechanism

To this end we performed molecular dynamics (MD) and free dynamics simulations. The basis for these calculations were the crystal structures of the protein in the on- and the off-state which represent the start and the final position of the light induced chromophore movement. By MD we simulated the two conceivable trans-cis photoisomerization pathways and also considered intramolecular properties of the chromophore adopting high-level quantum mechanical computations. As an outcome, these computer simulations showed that both the intrinsic chromophore properties, as well as the overall protein conformation, point to a so-called bottom hula twist mechanism as the predominant isomerization mechanisms in asFP595. In a hula twist mechanism, the two rings of the chromophore almost stay in place and only the methine bridge that connects both rings moves (Fig. 5). Such a hula twist is the most space efficient way to transform the trans- into a cis-conformation. In fact, the largest movement occurs at the phenolic oxygen of the chromophore, which translates by only $3 \cdot 10^{-10}$ meter. This infinitesimal movement, which also likely induces the already mentioned protonation event at the phenolic oxygen, seems to make all the difference between a fluorescent and a non-fluorescent asFP595.

Conclusion and Outlook

With this study we have elucidated the overall molecular light induced switching mechanism of the remarkable protein asFP595. It exhibits a complex photophysical behavior, so it may very well be anticipated that a more detailed understanding of its mechanism will bear even more surprises. Nonetheless, the obtained insight provides a solid framework of this molecular light switch for making it possible to lay out strategies for semi-rational approaches to engineer asFP595 variants with improved properties. Mutants of asFP595 with better switching properties and higher quantum efficiency may be used for applications ranging from high resolution optical microscopy to data storage.

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The team: From left to right.
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Bottom row: Gert Weber, Simon Trowitzsch, Martin Andresen, Christian Eggeling, Markus C. Wahl, Stefan W. Hell.