First Biphotochromic Fluorescent Protein moxSAASoti Stabilized for Oxidizing Environment

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Abstract

Biphotochromic proteins simultaneously possess reversible photoswitching (on-to-off) and irreversible photoconversion (green-to-red). High photochemical reactivity of cysteine residues is one of the reasons for the development of “mox”-monomeric and oxidation resistant proteins. Based on site-saturated simultaneous two points C105 and C117 mutagenesis we have chosen the C21N/C71G/C105G/C117T/C175A as the moxSAASoti variant, since its on-to-off photoswitching rate is higher, off-to-on recovery is more complete and photoconversion rates are higher than for the mSAASoti. We analyzed the conformational behavior of the F177 side chain by classical MD simulations. The conformational flexibility of the F177 side chain is mainly responsible for the off-to-on conversion rate changes and can be further utilized as a measure of the conversion rate. Point mutations in the mSAASoti mainly affect the pK_a values of the red form and the off-to-on switching. We demonstrate that the microscopic measure of the observed pK_a value is the C – O bond length in the phenyl fragment of the neutral chromophore. According to the molecular dynamic simulations with the QM/MM potentials, larger C – O bond lengths are found for proteins with larger pK_a . This feature can be utilized for prediction of the pK_a values of red fluorescent proteins.

Introduction

Since the decoding of the GFP gene, fluorescent proteins have become reliable and effective genetically encoded biological markers [1]. To date, a huge color palette of fluorescent proteins has been developed, covering the entire visible spectrum [2, 3]. Phototransformations of proteins have also been discovered: photoactivation [4, 5], reversible photoswitching [6], irreversible photoconversion [7]. Biphotochromic proteins simultaneously possessing reversible photoswitching and irreversible photoconversion were genetically engineered [8, 9, 10] and discovered in nature [11]. In recent years, work has been actively carried out to increase the chemical inertness of the proteins [12], stability at different pH values [13] and oxidative environmental conditions [14]. These factors can disrupt stable folding and maturation of the chromophore or lead to rapid photobleaching.

High photochemical reactivity of cysteine residues is one of the reasons for these problems moxFPs (“mox”-monomeric and oxidation resistant) proteins were developed [14–17]. In Table 1 key amino acid substitutions in mox-forms of obtained FPs are summarized.

Table 1
Amino acid substitutions in moxFPs.

FP	Ancestor FP	Amino acid substitution	Ref.
<i>secBFP2</i>	mTagBFP	C29A/C118S	[17]
<i>moxBFP</i>	EBFP2	C48S/C70V/V163A/V206K	[14]
<i>moxDendra2</i>	Dendra2	N41Q/C101A/C113T/C171A/N200Q	[15]
<i>moxMaple3</i>	mMaple3	C110V/N111Q/C180A/N227D	[16]
<i>moxNeonGreen</i>	mNeonGreen	C139S	[14]
<i>moxCerulean3</i>	mCerulean3	C48S/C70S/N105T/S30R/Y39N/I171V	[14]
<i>moxGFP</i>	SuperfolderGFP	C48S/C70S/V206K	[14]
<i>cfSGFP2</i>	SGFP2	C48S/C70M	[18]

The role of the chromophore-forming amino acid triplet is well known [19]. Amino acids in this triplet usually determine the fluorescent properties of a protein. The last amino acid in the triplet – glycine is absolutely conservative, because it is responsible for chromophore formation. The second amino acid is less conservative; in all natural GFP-like proteins this amino acid is tyrosine, but it can also be any other aromatic amino acid [20]. Tyrosine determines the correct chemical oxidation during chromophore maturation, preventing undesirable side reactions such as backbone fragmentation and hydrolysis [21].

The first amino acid in the chromophore is the most variable. It strongly determines the properties of a protein. Its substitution leads to a significant change in the properties of fluorescent proteins [22]. Thus, by replacing the first amino acid in the GFP and RFP chromophores, a multi-colored palette of fluorescent proteins was obtained [23, 24]. An interesting example is the mGeos family. This family of green PS proteins was obtained from the photoconvertible (*green-to-red*) protein mEos by replacing histidine in the HYG chromophore to the different amino acid residues [25]. Green-to-red photoconvertible proteins, starting with Kaede, has a HYG chromophore triplet, which provides an ability for green-to-red photoconversion under the 405 nm light illumination [26]. The C62H substitution led to the appearance of photoconversion properties in the PS Dronpa protein [10]. Despite the fact, that photoconvertible proteins phylogenetically located on the different branches, they show more than 50% identity to each other (Figure 1, Table S1).

At the same time for the wild genes no reversible photoswitching (*on-to-off*) was observed for the fluorescent proteins with the HYG chromophore until the fluorescent protein SAASoti was discovered [11], in which the HYG chromophore is capable of both irreversible photoconversion and reversible photoswitching of the green form without introducing genetic engineered substitutions to obtain these properties [27, 28] (Fig. S1).

Substitution F173S was discovered as leading to the appearance of the reversible photoswitching in photoconvertible proteins [8]. However, in SAASoti, phenylalanine occupies the corresponding position, which makes its structure unique and, apparently, significantly different from similar photoconvertible fluorescent proteins.

When we set a goal to obtain a monomeric and cysteine-free variant of SAASoti, it is important to note that the replacement of each amino acid residue of cysteine led to the unexpected effects, such as a shift in the pKa of the red form to the alkaline region, a change in the rate and depth of phototransformation [29], and an attempt to combine all the substitutions turned out to be a non-trivial task.

Results

As was described previously [29], the C21N/C71G/C175A mSAASoti (mSAASoti is a SAASoti with V127T substitution) form was characterized by the fastest green-to-red photoconversion and photobleaching of the green form rate, highest extinction coefficient (green form). The next aim was a development of the “mox” SAASoti form with all 5 cysteine residues substituted.

The structural role of cysteine residues and the effect of their substitutions on the properties of fluorescent proteins has not been sufficiently studied yet. In some cases mutation of cysteines to the other amino acid residues led to dim or dark proteins [15,17,30]. In some other phototransformable proteins (mMaple3 [16], Dendra2[15]) internal cysteines were substituted to valine and alanine, external – to serine and threonine. Based on alignment (Fig. 2), we tried different combinations of these substitutions for SAASoti (105 and 117 residues) and obtained dark proteins.

Using 3D alignment for analyzing position and orientation of C105 and C117 residues (Figure S2) we received, that for mSAASoti and Dendra2 they are the same. This makes obvious the important role of the interaction of cysteine residues with other amino acid residues, since the substitutions made in Dendra2 led to the production of bright and contrast moxDendra2 [15], while similar substitutions made in mSAASoti led to a violation of the protein folding and the chromophore maturation and the absence of fluorescence (data not presented).

In this case we use site-saturated two points mutagenesis for both substitutions simultaneously. The brightest clones contained glycine in position 105 and threonine or valine in position 117. New SAASoti mutants were expressed in *E.coli* cells and purified as described by standard scheme [28].

Since one of the substituted residues is a surface-facing one, its replacement could lead to increased aggregation of the new protein form. Therefore, oligomerization state of new mutants was analyzed by size-exclusion chromatography. Elution volume (Table S2) and elution profiles (Figure S2) for both mutants corresponded to the molecular weight of 25.7 kDa, which corresponded to a monomeric form of SAASoti. It could be evidence that external 117 residue does not play any important role in oligomerization of moxSAASoti.

The most important thing was to analyze how substitutions influenced on photochemical and photophysical properties. On-to-off green photoswitching kinetics are described by a bi-exponential model (Eq. (1)):

$$I = I_1 * \exp(-k_1t) + I_2 * \exp(-k_2t) + c \tag{1}$$

On-to-off switching rates for new variants are very similar and showed greater values than C21N/C71G/C175A mSAASoti and parental mSAASoti, but less photodestruction than C21N/C71G/C175A SAASoti. (Fig. 3)

C117V variant showed lower recovery after photoswitching than C117T (30% and 20% of the initial fluorescence intensity recovered after the first PS cycle in the case of C117T and C117V, respectively) and practically the same kinetic constants indicating on the high rates of “on-to-off”-switching (Table 2 and Fig. 3). The fluorescence recovery after second PS cycle is more complete, which probably indicates on the simultaneous photodestruction reaction in the first PS cycle. moxC117T shows the highest ratio of preexponential intensities, which indicates that the rapidly switching component (k_1) makes the maximum contribution to the overall kinetics.

Table 2. Kinetic parameters of “on-to-off”-switching for SAASoti variants calculated accorded to Eq. (1)

Variant	$k_1 \cdot 10^3, s^{-1}$	$k_2 \cdot 10^3, s^{-1}$	I_1/I_2
mSAASoti	55.5±1.0	8.3±0.7	1.9
C21N/C71G/C175A	95.2±2.0	5.0±0.2	0.7
moxC117T	131.6±0.9	7.5±0.3	3.7
moxC117V	113.6±1.3	7.3±0.4	2.9

We tested off-to-on thermal relaxation kinetics for moxSAASoti by changing of fluorescence at 520 nm. The relaxation kinetic constant value is 0.021±0.001 min⁻¹, which is up to 8 times greater than for other variants with substituted cysteines and of the same order as mSAASoti as previously published [29].

New data allows moxSAASoti to be define as a first “mox” negative **Reversibly Switchable Fluorescent Protein (RSFP)**. Negative RSFPs are the proteins, that in native state have chromophore in cis (“on”) conformation, and excitation light, that makes them fluorescence, meanwhile, at high power density of excitation light lead to *on-to-off* photoswitching [31]. We compared fluorescent properties, such as excitation and emission wavelengths, pKa, molecular brightness of moxSAASoti and well-known negative RSFPs (Table 3). As it can be seen, moxSAASoti is a quite bright RSFP. It outperforms the photo-switchable proteins of the GFP family, Dronpa3 and rsFastlime in brightness, but inferior to Gamillus (which was specially developed as acid-tolerant PSFP), and mGeos variants. Interesting to note, the properties of reversible photoswitching in the IrisFP-M159A protein were achieved by introducing

substitutions F173S in M159A, while in moxSAASoti there are phenylalanine and methionine residues in corresponding positions and it switches faster.

Table 3. Comparison of moxSAASoti and green negative RSFPs properties

RSFP	Chromo phore	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	pK _a	ϵ , M ⁻¹ * cm ⁻¹	ϕ	Brightness ($\phi*\epsilon$)
moxSAASoti	HYG	509/519	6.1	71 800	0.50	35.90
rsEGFP2 [32]	AYG	478/503	5.8	61 300	0.3	18.39
rsFolder2 [33]	AYG	478/503	5.5	44 000	0.23	10.12
Gamillus [34]	QYG	504/519	3.4	83 000	0.9	74.7
Dronpa3[35]	CYG	490/515	n.a.	58 000	0.33	19.14
rsFastLime [36]	CYG	496/518	n.a	39 094	0.77	30.1
mGeosC [25]	CYG	505/516	6.0	76 967	0.81	62.34
mGeosF [25]	FYG	504/515	5.0	53 135	0.85	45.16
mGeosM [25]	MYG	503/514	4.7	51 600	0.85	43.86
mGeosL [25]	LYG	501/513	5.0	53 448	0.72	38.48
mGeosE [25]	EYG	501/513	6.0	69 630	0.75	52.22
mGeosS [25]	SYG	501/512	5.0	64 602	0.76	49.1
IrisFP-M159A [37]	HYG	484/513	4.7	62 800	0.18	11.3

We tested the green to red photoconversion capacity of new variants. Mox variants like their closest ancestor C21N/C71G/C175A showed high green-to-red photoconversion rate, but low degree of photoconversion (Fig 4). We assume several possible reasons for this fact: 1) low brightness of the red form; 2) unstable red form. It is certainly could be combination of all phenomena, which is the subject of further research.

Process of photoconversion was detected by changes in red form fluorescence intensity (λ_{em} =590 nm). It can be described by a bi-exponential model (Eq. (2)):

$$I = -I_1 * \exp(-k_1t) + I_2 * \exp(-k_2t) + c \tag{2}$$

where the first exponent is responsible for the red form formation, while the second exponent describes its photodestruction, c – background and residual signal. The corresponding kinetic parameters (Table 4) for mox variants are close to the parent C21N/C71G/C175A SAASoti, which could indicate that residues in positions 105 and 117 do not affect the green-to-red photoconversion.

Table 4. Kinetic parameters of green to red photoconversion, calculated for SAASoti mutants according to Eq. (2)

SAASoti form	$k_1 \cdot 10^3, s^{-1}$	$k_2 \cdot 10^3, s^{-1}$
mSAASoti	62 ±1	55±1
C21N/C71G/C175A	273±16	106±4
moxC117T	278±16	79±2
moxC117V	270±14	107±4

Based on photochemical and photophysical properties of new mox forms of SAASoti protein, we have chosen C21N/C71G/**C105G/C117T**/C175A variant, since its on-to-off photoswitching rate is higher, off-to-on recovery is more complete and photoconversion rates are higher than mSAASoti.

We compared main physicochemical properties (excitation/emission maxima $\lambda_{ex}/\lambda_{em}$, pK_a values of the chromophore, molar extinction coefficient (ϵ) and quantum yield (ϕ)) of moxSAASoti and other mSAASoti mutants with cysteine substitutions (Table 5). Interestingly, that most impact new substitutions provided upon pK_a of the moxSAASoti red form. Previously, all cysteine substitutions resulted in higher pK_a values (except SAASoti C117S, that has the same as mSAASoti pK_a), we suggest it could be allosteric effect from T117, because previously mutant with single C117S substitution showed the lowest pK_a of the red form of all other mutants with single substitutions of cysteine residues.

Table 5. Physicochemical properties and fluorescent parameters of different mSAASoti mutant forms.

	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	pK _a	ϵ , M ⁻¹ * cm ⁻¹	ϕ	Brightness ($\phi*\epsilon$)
mSAASoti	509/519	6.3±0.1	75.0	0.59±0.02	44.3
	573/579	6.6±0.1	24.0		
C21N	509/519	6.4±0.1	82.4	0.61±0.02	50.3
	579/590	7.5±0.1			
C105V	509/519	6.5±0.1	61.0	0.60±0.02	36.6
	576/589	7.1±0.1			
C71V	509/519	6.5±0.1	65.1	0.63±0.04	41.0
	577/590	7.0±0.1			
C175A	509/519	6.7±0.1	80.1	0.55±0.05	44.0
	580/587	7.8±0.1			
C117S	509/519	6.2±0.1	66.3	0.54±0.03	35.8
	580/590	6.7±0.1			
C21N/C71V	509/519	6.3±0.1	48.9	0.58±0.02	28.4
	577/589	7.0±0.1			
C21N/C175A	509/519	6.3±0.1	65.4	0.55±0.03	36.0
	580/590	7.4±0.1	12.7		
C21N/C71G/C175A	509/519	6.4±0.1	83.8	0.60±0.02	50.3
	577/589	7.2±0.2	14.8		
moxSAASoti	509/519	6.1±0.1	71.8	0.50±0.02	35.9
	577/589	6.3±0.1	11.3		

Point mutations in the mSAASoti mainly affect the pK_a values (Table 5) of the red form and the off-to-on switching. Noteworthy, single and triple mutations that are presented in the moxSAASoti considerably changes these two macroscopic properties. However, substitutions of all cysteine residues result in the recovery of the mSAASoti properties. It is not evident, which particular microscopic structural features are responsible for the changes of the macroscopic parameters. Previously [29] it was demonstrated that the flexibility of the F177 determines the rate of the off-to-on conversion and that change of the C-O distance of the phenyl fragment of the chromophore is responsible for the pK_a shift. Here we test these notions on the set of five proteins of the mSAASoti family: the mSAASoti, its single mutants C21N and C175A and a

triple mutant C21N/C71G/C175A with exactly the same point mutations as in the moxSAASoti, and moxSAASoti itself.

We performed 200 ns classical MD run for each model system and analyzed the conformational behavior of the F177 side chain. As the measure of the conformational diversity, we choose the dihedral angle C-C_α-C_β-C_γ (Figure 5 A,B). The mSAASoti and moxSAASoti variants predominantly demonstrate conformations with the dihedral values between 140 and 180 degrees. For slower proteins, single and triple mutants of mSAASoti, the dihedrals are distributed between 20 and 100 degrees. This conformation is less favorable for the off-to-on conversion as the chromophore binding pocket is tighter in this case that hinders isomerization (Figure 5). Thus, the conformational flexibility of the F177 side chain is mainly responsible for the off-to-on conversion rate changes and can be further utilized as a measure of the conversion rate.

The C-O bond lengths in the substituted phenols are known to correlate with their pK_a values [38]. Here we utilize this approach for more complicated systems, the same chromophore in slightly different protein environments due to point mutations. We perform QM/MM MD runs of the models comprising neutral red chromophore and analyze d(C-O) distributions (Figure 5 C). Even though, the chromophores in fluorescent proteins have a large number of interatomic interactions, still the elongation of the C-O distance, being a calculated parameter, reproduces the experimentally observed increase of the pK_a value (Figure 5 D). This result is of great importance as it demonstrate that even though we cannot distinguish the impact of each amino acid residue and its substitution, we can evaluate the d(C-O) values along the MD trajectories and use it as a calculated parameter to predict the pK_a value.

Materials And Methods

Mutagenesis and colonies screening

Site-saturated mutagenesis was performed by overlapped PCR with degenerate primers (Table 6). PCR reactions were carried out sequentially with each pair of primers using Pfu DNA polymerase. DNA with random substitutions in two positions (105 and 117) was cloned into pEt22b vector and transformed into *E. coli* BL21(DE3) cells. The resulting colonies were transferred on LB-agar medium with IPTG and grown at 20 ° C for 24 hours. Colonies were analyzed for fluorescence on Olympus CKX41SF microscope by irradiation with excitation light at 470 nm.

Table 6. Degenerate primers for overlapped PCR.

Primer	Sequence
C105X_fw	GAT GGC GGA TTT NNN ACA GTC AGT GCA
C105X_rev	TGC ACT GAC TGT NNN AAA TCC GCC ATC
C117X_fw	GAC AAC NNN TTC ATT CAC ACA TCC ATG
C117X_rev	TGT GTG AAT GAA NNN GTT GTC TTT AAG TTT TAT

Protein expression and purification. moxSAASoti was expressed and purified as described previously [28], with exception that cells were disrupted by ultrasonication. *Oligomerization analysis* by size-exclusion chromatography was performed as described earlier [28]. *Absorbance and fluorescence spectra* were detected using Cary 60 and Cary Eclipse respectively as described earlier [28]. *Green-to-red photoconversion and reversible photoswitching* experiments were performed using a self-made setup based on Olympus CX41 upright microscope with Thorlabs (USA) light sources, 400 nm (and 560 nm for red form excitation) for photoconversion, 470 nm for photoswitching using Avesta ASP-75 spectrometer for detection. LEDs light was passed through the bandpass filters Thorlabs MF390/18 and Chroma ET470/24m. We obtained 282.4, 706.1 mW/cm² maximum power densities respectively after Olympus 20x/0.4 Plan N objective. Protein solutions were placed in 5 ml glass capillary. *Thermal relaxation* was analyzed by recording fluorescence intensities at 520 nm with 479 nm excitation. Intensities were recorded every 10 minutes to $I=1/2I_{\max}$ values. *Data analyzes* of all phototransformation experiments were performed with OriginPro 2018 software package.

Classical MD simulations

Additionally, we prepared the triple C21N/C71G/C175A mutant and the mox form. The CHARMM36 [39] force field parameters were utilized for protein and the CGenFF [40] force field parameters for the chromophore in the green form. The system was solvated in the rectangular water box with the TIP3P [41] water molecules and neutralized by adding sodium ions. Classical molecular dynamics simulations were performed in the NAMD software package [42]. Each system was preliminary equilibrated by 10000 minimization steps and 20 ns MD run. Production runs for the WT and C21N variants of SAASoti were performed for 200 ns with 1 fs time step in the NPT ensemble at $p = 1$ atm and $T = 300$ K. The pressure and temperature were controlled by Nosé-Hoover barostat and Langevin thermostat, respectively. To decrease the influence of error accumulation for such long trajectories, we randomly reassigned velocities every 40 ns. The cutoff distances were 12 Å for both electrostatic and van der Waals interactions with switching to the smoothing function at 10 Å.

MD simulations with QM/MM potentials

The systems for the QM/MM (combined quantum mechanics / molecular mechanics) MD simulations were preliminary equilibrated in classical MD runs as described above. The simulations were performed for the same set of five systems and chromophore was in the neutral red form. The MM subsystems were described similarly to the classical MD. The QM part was composed of the chromophore, the side chains

of Gln42, Thr63, Arg70, Arg95, Ser146, His197, Glu213 (in the neutral form) and two water molecules. The green to red conversion was manually performed and these coordinates were used as initial for the QM/MM MD runs. The system was preliminary minimized for 100 steps. After that the 5 ps production runs were performed. The QM part was described at the PBE0-D3/6-31G** Kohn-Sham DFT level [43,44]. The QM/MM MD simulations were performed using the interface [45] for the classical MD software NAMD [42] and a quantum chemistry package TeraChem [46].

Declarations

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Contributions: N.K.M- manuscript preparation, molecular cloning, analyzed experimental data, Figures 1-4, Tables 1-4. M.G.K.- MD simulations and MD simulations with QM/MM potentials, Figure 5. A.V.G experimental design, Table 5. I.D.S- setup for studying phototransformations, design of experiments on phototransformations and data processing. A.P.S. supervised the project.

All authors discussed the data and contributed to the manuscript revisions.

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Figures

Figure 1

(A) Bayesian phylogenetic tree of the green-to red photoconvertible proteins. (Data obtained with Clustal Omega tool <https://www.ebi.ac.uk/Tools/services/rest/clustalo>. (B) Photochromic properties, oligomerization state and parental gene. BPh – biphotochromic protein, PC -photoconvertible protein, PS – photoswitchable protein.

	10	20	30	40	50	
mSAASoti	-----MALSKQYIPDDMELIFHMDGCVNGHYFTIVATGKAKPYEGKQNLKATVTKGAPLP					
moxDendra2	----MNTPGINLIKEDMRVKVHMEGNVNGHAFVIEGEGKGKPYEGTQTAQLTVKEGAPLP					
Dendra2	----MNTPGINLIKEDMRVKVHMEGNVNGHAFVIEGEGKGKPYEGTQTANLTVKEGAPLP					
NijiFP	----MNTPGINLIKEDMRVKVHMEGNVNGHAFVIEGEGKGKPYEGTQTANLTVKEGAPLP					
pcDronpa	-----MSVIKPDPMKIKLRMEGAVNGHPFAIEGVGLGKPFEGKQSM DLKVKEGGPLP					
moxMaple3	MVSKGEETIMSVIKPDPMKIKLRMEGNVNGHAFVIEGEGSGKPFEGIQ TIDLEVKEGAPLP					
mMaple3	MVSKGEETIMSVIKPDPMKIKLRMEGNVNGHAFVIEGEGSGKPFEGIQ TIDLEVKEGAPLP					
IrisFP	-----MSAIKPDPMKINLRMEGNVNGHHFVIDGDGTGKPFEGKQSM DLEVKEGGPLP					
moxEos3.2	-----MVSAIKPDPMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSM DLEVKEGGPLP					
	60	70	80	90	100	110
mSAASoti	FSTDILSTVMHYGNRCIVHYPPGIPDYFKQSFPEGYSWERTFAFEDGGFCTVSADIKLKD					
moxDendra2	FSYDILTAVHYGNRVFTKYPEDIPDYFKQSFPEGYSWERTMTFEDDKGIATIRSDISLEG					
Dendra2	FSYDILTAVHYGNRVFTKYPEDIPDYFKQSFPEGYSWERTMTFEDDKGICTIRSDISLEG					
NijiFP	FSYDILTAVHYGNRVFTKYPEDIPDYFKQSFPEGYSWERTMTFEDDKGICTIRSDISLEG					
pcDronpa	FAYDILTAFHYGNRVFAKYPENIVDYFKQSFPEGYSWERSMSYEDGGICIATNDITLDG					
moxMaple3	FAYDILTAFHYGNRVFTKYPRKIPDYFKQSFPEGYSWERSMTYEDGGIVQATNDITMEE					
mMaple3	FAYDILTAFHYGNRVFTKYPRKIPDYFKQSFPEGYSWERSMTYEDGGICNATNDITMEE					
IrisFP	FAFDILTAFHYGNRVFAEYPDHIQDYFKQSFPKGYSWERSLTFEDGGICIARNDITMEG					
moxEos3.2	FAFDILTAFHYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGIANARNDITMEG					
	120	130	140	150	160	170
mSAASoti	NCFIHTSMFHGTNFPADGPVMQRKTIQWEKSIEKMTVSDGIVKGDITMFLLEGGGKYRC					
moxDendra2	DTFFQNVRFKGTNFPNGPVMQKKTLLKWEPESTEKLHVRDGLLVGNINMALLLEGGGHYLA					
Dendra2	DCFFQNVRFKGTNFPNGPVMQKKTLLKWEPESTEKLHVRDGLLVGNINMALLLEGGGHYLC					
NijiFP	DCFFQNVRFKGTNFPNGPVMQKKTLLKWEPESTEKLHVRDGLLVGNINMALLLEGGGHYLC					
pcDronpa	DCYIYEIRFDGVNFPANGPVMQKRTVKWEPESTEKLYVRDGVVLKGDVNMALSLGGGHYRC					
moxMaple3	DSFINKIHFKGTNFPNGPVMQKRTVGWEVSTEKMYVRDGVVLKGDVMMKLLLKGGSHYRA					
mMaple3	DSFINKIHFKGTNFPNGPVMQKRTVGWEVSTEKMYVRDGVVLKGDVMMKLLLKGGSHYRC					
IrisFP	DTFYNKVRFHGVNFPANGPVMQKKTLLKWEPESTEKMYVRDGVLTGDITMALLLEGNAHYRC					
moxEos3.2	DTFYNKVRFYGTNFPANGPVMQKKTLLKWEPESTEKMYVRDGVLTGDIEMALLLEGNAHYRA					
	180	190	200	210	220	
mSAASoti	QFHTSYKAK-KVVEMPQSHYVEHSIERTNDDG--TQFELNEHAVARLNEI-----					
moxDendra2	DFKTTYKAK-KVVQLPDAHFDHRIEILGQSDSYNKVKLYEHAVARYSPLPSQVW--					
Dendra2	DFKTTYKAK-KVVQLPDAHFDHRIEILGNDSDYNKVKLYEHAVARYSPLPSQVW--					
NijiFP	DSKTTYKAK-KVVQLPDAHFDHRIEILGNDSDYNKVKLYEHAVARYSPLPSQVW--					
pcDronpa	DFKTTYKAK-KVVQLPDYHFVDHIEIKSHDKDYSNVNLHEHAEAHSGLPQAK---					
moxMaple3	DFRTTYKVKQKAVKLPKAHFVDHRIEILSHDKDYNKVKLYEHAVARNSTDMSDELYK					
mMaple3	DFRTTYKVKQKAVKLPKAHFVDHRIEILSHDKDYNKVKLYEHAVARNSTDMSDELYK					
IrisFP	DSRTTYKAKEKGVKLPGYHLVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR--					
moxEos3.2	DFRTTYKAKEKGVKLPGAHFVDHAIEILSHDKDYNKVKLYEHAVAHSGLPDNARR--					

Figure 2

Sequence alignment of “mox” photoconvertible proteins, biphotochromic proteins and mSAASoti. The numbering is based on mSAASoti. Cysteine residues to mutate are highlighted in grey, chromophore is indicated by black shade, 177 residue is bold and underlined.

Figure 3

Normalized photoswitching profiles of mSAASoti variants. “Off”-switching induces by 470 nm illumination, “on”-switching by 400 nm illumination for 5 seconds. Proteins dissolved in 20 mM NaHCO₃ buffer, pH 9,2

Figure 4

Green-to-red photoconversion of SAASoti variants induced by 400 nm combined with 550 nm illumination. Protein samples prepared in 200 mM Tris-HCl, pH 7,4.

Figure 5

(A) Distributions of the C-C α -C β -C γ dihedral of the Phe177 in the mSAASoti, its C21N, C175A and C21N/C71G/C175A mutants and moxSAASoti. (B) The chromophore and two different conformations of the F177 residue, the conformation colored cyan correspond to the faster off-to-on isomerization and magenta – to the slower off-to-on one. The inset shows the color code for the data for different mSAASoti variants on panels A, C and D. (C) Distributions of the C-O bond distances in the phenyl fragment of the neutral red form of the chromophore for different mSAASoti variants. (D) The relation between the calculated shifts of the mean values of the $\text{d}(\text{C}-\text{O})$ and experimental pKa shifts relative to the mSAASoti variant

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