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Absorption and emission spectroscopic characterization of photo-dynamics of photoactivated adenylyl cyclase mutant bPAC-Y7F of *Beggiatoa* sp.

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ABSTRACT

The photoactivated cyclase bPAC of the microbial mats bacterium *Beggiatoa* sp. consists of a BLUF domain and an adenylyl cyclase domain. It has strong activity of photo-induced cyclic adenylyl monophosphate (cAMP) formation and is therefore an important optogenetic tool in neuroscience applications. The SUMO-bPAC-Y7F mutant where Tyr-7 is replaced by Phe-7 in the BLUF domain has lost the typical BLUF domain photo-cycle dynamics. Instead, the investigated SUMO-bPAC-Y7F mutant consisted of three protein conformations with different triplet based photo-dynamics: (i) reversible flavin quinone (Fl) cofactor reduction to flavin semiquinone ($FlH^{\cdot}$), (ii) reversible violet/near ultraviolet absorbing flavin photoproduct (FlA) formation, and (iii) irreversible red absorbing flavin photoproduct (FlC) formation. Absorption and emission spectroscopic measurements on SUMO-bPAC-Y7F were carried out before, during and after light exposure. Flavin photo-dynamics schemes are developed for the SUMO-bPAC-Y7F fractions performing photo-induced $FlH^{\cdot}$, FlA, and FlC formation. Quantitative parameters of the flavin cofactor excitation, relaxation and recovery dynamics in SUMO-bPAC-Y7F are determined.

1. Introduction

Recently, a novel photoactivated adenylyl cyclase, bPAC (synthetic construct BLUF domain regulated adenylyl cyclase, gene accession number GU461307), consisting of a BLUF (Blue-Light sensor Using Flavin) domain and a cyclase homology domain (CHD) from the filamentous sulfur-oxidizing bacterium *Beggiatoa* sp. ([1–3] and reference therein) was expressed heterologously and characterized by electrophysiology and optical spectroscopy [4]. The cyclase activity of light-dependent ATP to cAMP conversion was studied in *Escherichia coli*, oocytes from *Xenopus laevis*, hippocamal slice cultures from Winstar rats, and brains dissected from transgenic *Drosophila* flies [4]. The light induced cellular second messenger cAMP modulation in neuronal cells and tissues makes bPAC a promising optogenetic tool for cell biology and neuroscience ([5–7] and references therein).

The bPAC light-induced cyclase activity was tailored by specific amino acid mutations in the BLUF domain of bPAC [5,8,9]. The spectral response and the temporal response as well as the light-induced ATP to cAMP conversion efficiency could be varied within certain ranges by BLUF domain amino acid mutations [9]. The mutation of Tyr-7 to Phe-7 caused changes in the BLUF domain and the cyclase domain behavior: (i) the cAMP activity of bPAC-Y7F in the dark was a factor of 15 stronger than for bPAC in the dark. The cAMP light activity of bPAC increased a factor of 17 compared to the dark, while in bPAC-Y7F the cAMP light activity reduced to a factor of 0.7 compared to the dark. (ii) Blue light exposure of bPAC resulted in typical BLUF-type photo-cycling, while blue light exposure of bPAC-Y7F resulted in reversible flavin cofactor reduction from fully oxidized flavin (flavin quinone) to semi-reduced flavin (flavin semiquinone) [8].

A detailed study of the photo-dynamics of the BLUF domain of bPAC-Y7F is given in this paper to enlighten the changes in photo-activation caused by the mutation of Tyr-7 to Phe-7. Absorption and emission spectroscopic measurements on bPAC-Y7F were carried out before, during, and after light exposure. The results show that the singlet excited-state based BLUF domain photo-cycle dynamics [10–13] is lost. Photo-induced reversible flavin quinone cofactor reduction to flavin semiquinone $FlH^{\cdot}$, reversible violet/UVA absorbing photoproduct formation FlA, and irreversible red light absorbing photoproduct formation FlC were observed. The analysis of the observed photo-dynamics reveals the presence of three bPAC-Y7F protein conformations. Flavin photo-dynamics

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schemes are developed for them and applied to extract quantitative parameters of the excitation, relaxation and recovery dynamics in bPAC-Y7F.

2. Experimental

2.1. Sample preparation

The expression of SUMO-bPAC and Y7F mutant was described in [8] (SUMO = small ubiquitine-related-modifier 1, Homo sapiens, UniProt # P63165). The amino acid sequence of SUMO-bPAC-Y7F is shown in Fig. 8b. The bPAC domain is indicated by Italian letters (350 aa, from 124 to 473). The BLUF domain is high-lighted by bold italic letters and grey underlay (from 129 to 215), and the cyclase homology domain is high-lighted by white italic letters in black background (from 226 to 451). The molar mass of SUMO-bPAC-Y7F is $M_{\mathrm{mol,SUMO\text{-}bPAC\text{-}Y7F}} = 53589.52\ \mathrm{g\ mol^{-1}}$. The mutation of Y7 to F7 was achieved with the aid of a QuikChange® II XL site-directed mutagenesis kit (Stratagene) using the primers given in [8].

2.2. Spectroscopic characterization

SUMO-bPAC-Y7F samples in pH 7.5 phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol) were stored in a refrigerator at −80 °C. Before usage the samples were thawed and then stored in the dark at 4 °C. Measurements were carried out in fused silica ultra-micro cells (inner size $1.5 \times 3 \times 5\ \mathrm{mm^3}$) at room temperature (20.5 °C).

Absorption measurements were carried out with a spectrophotometer (Cary 50 from Varian). Transmission spectra $T(\lambda)$ were recorded, and absorption coefficient spectra $\alpha(\lambda)$ were calculated using the relation $\alpha(\lambda) = -\ln[T(\lambda)]/\ell$, where ℓ is the sample length.

The absorption spectra development of the samples during blue light exposure was studied in the spectrophotometer by transverse sample exposure (path length $\ell_{\mathrm{exc}} = 1.5$ mm) at 455 nm with a LED light source (LEDC1 from Thorlabs) and repetitive transmission spectra recording. The excitation intensity was measured with a power meter (model PD 200-UV-SH photodiode detector head with NOVA power monitor from Ophir). After excitation light switch-off the absorption spectra development was followed by transmission spectra measurement at certain time intervals.

Fluorescence emission spectra, $S_F(\lambda)$, and fluorescence excitation spectra, $S_{\mathrm{ex}}(\lambda)$, were recorded with a fluorimeter (Cary Eclipse from Varian) under magic angle conditions (vertical polarized excitation, polarizer in detection path oriented under an angle of 54.7° to the vertical) [14], parallel polarization conditions (vertical polarized excitation and polarizer in fluorescence path oriented for vertical polarized light passage), and perpendicular polarization conditions (vertical polarized excitation and polarizer in fluorescence path oriented for horizontal polarized light passage). In all cases the excitation light path was perpendicular to the fluorescence detection light path (90° angle arrangement). All spectra were corrected for the spectral sensitivity of the spectrometer and the photo-detector [15–17].

Fluorescence quantum distributions [18], $E_F(\lambda)$, fluorescence excitation quantum distributions [16], $E_{\mathrm{ex}}(\lambda)$, fluorescence emission polarization distributions [14], $p_F(\lambda)$, and fluorescence excitation polarization distributions, $p_{\mathrm{ex}}(\lambda)$, were determined ($p = (S_{\parallel} - S_{\perp})/(S_{\parallel} + S_{\perp})$, $S_{\parallel}$ and $S_{\perp}$ are parallel and perpendicular polarized signals). The dye riboflavin in aqueous solution at pH 7 (fluorescence quantum yield $\phi_{F,\mathrm{ref}} = 0.26$ [19,20]) was used as reference standard for fluorescence quantum distribution calibration. The fluorescence quantum yield is given by $\phi_F = \int_{em} E_F(\lambda) d\lambda$, where the integration runs over the emission wavelength region.

Fluorescence lifetime measurements were carried out with second harmonic light pulses of a mode-locked titanium sapphire laser system (Hurricane from Spectra-Physics, pulse duration $\Delta t_P \approx 3$ ps, wavelength $\lambda_P = 400$ nm) and a micro-channel-plate photomultiplier (Hamamatsu type R1564U-01) connected to a fast digital oscilloscope (LeCroy type 9362). The time resolution of the detection system was approximately 500 ps.

3. Results

3.1. Absorption spectroscopic characterization

3.1.1. Unexposed SUMO-bPAC-Y7F

The absorption coefficient spectrum $\alpha(\lambda)$ of a SUMO-bPAC-Y7F sample is shown by the thick dotted curve in Fig. 1a. In the wavelength range above 310 nm the absorption is dominated by fully oxidized flavin (Fl). Below 310 nm flavin and bPAC apo-protein with SUMO extension protein (in total 15 Tyr and 2 Trp) contribute to the absorption. The S_0–S_1 absorption band of Fl peaking at 438 nm and the S_0–S_2 absorption band of Fl with absorption maximum at 374 nm exhibit vibronic structure, indicating an ordered localization of the flavin cofactor in the BLUF domain binding pocket.

The absorption coefficient at $\lambda = 445$ nm was applied to determine approximately the flavin concentration. The flavin number density was obtained by the relation $N_{\mathrm{Fl}} = \alpha(445\ \mathrm{nm})/\sigma_{\mathrm{Fl}}(445\ \mathrm{nm}) \approx 2.26 \times 10^{16}\ \mathrm{cm^{-3}}$ using $\alpha(445\ \mathrm{nm}) = 1.05\ \mathrm{cm^{-1}}$ and $\sigma_{\mathrm{Fl}}(445\ \mathrm{nm}) = 4.65 \times 10^{-17}\ \mathrm{cm^2}$ [21] ($C_{\mathrm{Fl}} = 3.75 \times 10^{-5}\ \mathrm{mol\ dm^{-3}}$).

3.1.2. Blue-light exposed SUMO-bPAC-Y7F

The influence of blue-light exposure on the absorption of SUMO-bPAC-Y7F is seen in Fig. 1a. The sample was excited for $t_{\mathrm{exp}} = 84$ s at 455 nm with an incident intensity of $I_{\mathrm{exc}} = 0.18\ \mathrm{W\ cm^{-2}}$. The top part shows the absorption coefficient spectra for different exposure times, t_{exp}, and corresponding exposed input energy densities, w_{exp}. The bottom part shows the corresponding difference absorption coefficient spectra $\Delta\alpha(\lambda, w_{\mathrm{exp}}) = \alpha(\lambda, w_{\mathrm{exp}}) - \alpha(\lambda, w_{\mathrm{exp}} = 0)$. Positive values indicate absorption increase due to light exposure and negative values indicate absorption decrease due to light exposure. The conversion of oxidized flavin Fl to flavin semiquinone FlH· is clearly seen (absorption rise in range from 480

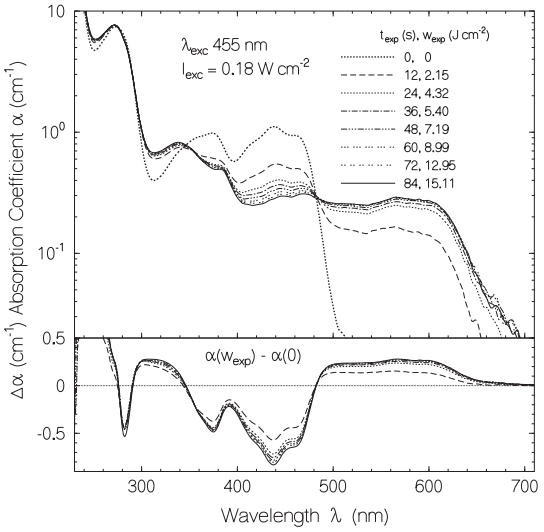

Fig. 1a. Top part: Absorption spectra development of SUMO-bPAC-Y7F due to blue light exposure. Excitation wavelength $\lambda_{\mathrm{exc}} = 455$ nm, excitation intensity $I_{\mathrm{exc}} = 0.18\ \mathrm{W\ cm^{-2}}$. Exposure time values t_{exp} and input excitation energy densities w_{exp} are given in the figure legend. Bottom part: Absorption difference spectra, $\Delta\alpha(\lambda, w_{\mathrm{exp}}) = \alpha(\lambda, w_{\mathrm{exp}}) - \alpha(\lambda, w_{\mathrm{exp}} = 0)$, for curves displayed in the top part.