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Absorption and emission spectroscopic characterization of blue-light receptor Slr1694 from *Synechocystis* sp. PCC6803

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Abstract

The BLUF protein Slr1694 from the cyanobacterium *Synechocystis* sp. PCC6803 is characterized by absorption and emission spectroscopy. Slr1694 expressed from *E. coli* which non-covalently binds FAD, FMN, and riboflavin (called $Slr1694_I$), and reconstituted Slr1694 which dominantly contains FAD (called $Slr1694_{II}$) are investigated. The receptor conformation of Slr1694 (dark adapted form $Slr1694_r$) is transformed to the putative signalling state (light adapted form $Slr1694_s$) with red-shifted absorption and decreased fluorescence efficiency by blue-light excitation. In the dark at 22 °C, the signalling state recovers back to the initial receptor state with a time constants of about 14.2 s for $Slr1694_I$ and 17 s for $Slr1694_{II}$. Quantum yields of signalling state formation of approximately 0.63 ± 0.07 for both $Slr1694_I$ and $Slr1694_{II}$ were determined by transient transmission measurements and intensity dependent steady-state transmission measurements. Extended blue-light excitation causes some bound flavin conversion to the hydroquinone form and some photo-degradation, both with low quantum efficiency. The flavin-hydroquinone re-oxidizes slowly back (time constant 5–9 min) to the initial flavoquinone form in the dark. A photo-cycle dynamics scheme is presented.

Keywords: BLUF domain; Slr1694 protein from *Synechocystis* sp. PCC6803; Blue-light photoreceptor; Absorption spectroscopy; Fluorescence spectroscopy; Photo-cycle; Photo-reduction; Flavoprotein; Flavins; FAD; FMN; Riboflavin

1. Introduction

The blue-light response of biological organisms is an active field of research (for recent books see [1–3]). There are three classes of blue-light receptors containing flavin cofactors, the cryptochromes with sensing cry domains, the LOV (Light Oxygen Voltage) domains first discovered in the family of phototropin proteins (phot), and the BLUF (sensors of blue light using FAD) domains. The sensing domains non-covalently bind flavin-adenine-dinucleotide (FAD) in the case of cry domains and BLUF domains, and flavin-mononucleotide (FMN) in the case of LOV domains. The BLUF domains studied thus far *in vitro* non-specifically bind flavins, i.e. FAD, FMN and riboflavin.

BLUF domains were found in different sensing proteins. These are AppA from the phototrotophic proteobacterium *Rhodobacter sphaeroides* (BLUF domain AppA) [4–19], YcgF in *Escherichia coli* (BLUF domain Blrp) [20], BlrB from *Rhodobacter sphaeroides* (BLUF domain BlrB) [20,21], Tll0078 of the thermophilic unicellular cyanobacterium *Thermosynechococcus elongates* BP-1 [23–25], Slr1694 from the cyanobacterium *Synechocystis* sp. PCC6803 [25–30], PAC_α (photo-activated adenylyl cyclase) from the unicellular flagellate *Euglena gracilis* (BLUF domain F2) [16,31–34], and at least 15 other flavoproteins in micro-organisms [35]. Blue-light excitation of BLUF domains (receptor state) leads to a slight red-shift of the absorption band (formation of putative signalling state) and a recovery to the initial absorption behaviour in the dark [4–8,23,24,26,31].

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The 17 kDa Slr1694 protein of cyanobacterium *Synechocystis* sp. PCC6803 is encoded by an orf *slr1694* gene [35]. The amino acid sequence of Slr1694 is given in [35] and is found under http://www.expasy.org/Cgi-bin/niceprot.pl/. Its BLUF domain retains non-covalently associated FAD and is involved in photo-taxis events [25,36]. Light-induced structural changes of the Slr1694 BLUF domain were observed (spectral red-shift) [26–28]. It was found that Slr1694 exists in solution in oligomeric form (trimer or tetramer) [26]. A photo-cyclic behaviour with signalling state formation by rearrangement of hydrogen bonding and fast dark recovery was found by light-induced Fourier transform infrared (FTIR) difference spectroscopy [25–28]. A change of hydrogen bonding strength at C4=O of FAD was identified as being responsible for the UV–vis red-shift [25,27]. When Tyr8 in the BLUF domain was replaced by phenylanilin the visible spectral red-shift and the absorption band changes of the C4=O stretching vibration disappeared, indicating that Tyr8 is responsible for the photo-cycle (C4=O hydrogen bonding causes spectral red-shift) [25]. A detailed model of photo-induced hydrogen-bond switching through a radical pair mechanism involving Tyr8 to flavin electron-transfer, transient $FAD^{\cdot -}$ and subsequent $FADH^{\cdot}$ formation, Gln50 rotation by 180°, and radical-pair recombination between $FADH^{\cdot}$ and Tyr8 is derived from femtosecond transient absorption spectroscopy in [30].

In this paper the full-length protein Slr1694 (151 amino acids, molar mass 17 kDa determined by SDS-PAGE) from the cyanobacterium *Synechocystis* sp. PCC6803 containing the Slr1694 BLUF domain (from amino acid 3 to amino acid 94) is characterized by absorption and emission spectroscopy. We studied the protein as expressed from *E. coli* (called $Slr1694_I$), binding the flavins FAD (flavin-adenine-dinucleotide), FMN (flavin-mononucleotide), and riboflavin. We also studied the Slr1694 protein that had been reconstituted with FAD (called $Slr1694_{II}$), thus containing dominantly FAD as cofactor (ca. 91%) thereby coming nearer to the natural situation, since *in vivo* the cofactor of Slr1694 is FAD. The flavin chromophore composition was analysed by HPLC. The fluorescence quantum yields and fluorescence lifetimes of the proteins in the dark-state and in the light-adapted state were determined. The photo-cycle dynamics of signalling state formation from the receptor state by light exposure, and of the dark recovery of the signalling state to the receptor state at room temperature are studied. The quantum yields of photo-induced signalling state formation are determined. The presence of a small amount of non-bound free flavin is revealed, and the photo-degradation of free flavin to lumichrome and lumiflavin derivatives [37] is observed. A low-efficient photo-reduction of flavin in the signalling state of Slr1694 to flavin-hydroquinone, and a re-oxidation back to the initial dark-adapted form occurs. Also low-efficient irreversible photo-degradation of non-covalently bound flavin in Slr1694 is observed. A photo-cyclic scheme is derived.

2. Experimental

2.1. Sample preparation

Slr1694-DNA encoding the full-length Slr1694-protein (aa 1–151, Acc No.: D90913, NP_441709), was amplified by polymerase chain reaction (PCR) from *Synechocystis* sp. PCC6803 DNA (kindly provided by Dr. I. Maldener, Regensburg) and cloned between *Eco*RI and *Hin*dIII sites of the pET28a(+) vector (Invitrogen, Karlsruhe, Germany). The protein was expressed in *E. coli* strain BL21(DE3)pLysS overnight at 18 °C, 0.7 mM IPTG (isopropyl-β-thiogalactopyranosid). Slr1694 was purified on Ni-NTA-resin (Quiagen, Hilden Germany) according to the supplier's instruction. The eluate was dialysed 2 times against 200 volumes of 10 mM NaPi, 10 mM NaCl, pH 8.0, and concentrated by ultrafiltration (Amicon Ultra-15, M.W.C.O. 10000, Millipore). The obtained protein is called $Slr1694_I$ in this paper.

For the preparation of $Slr1694_{II}$, the Slr1694 apoprotein was prepared by dialysis of a concentrated $Slr1694_I$ sample against 3×3000 volumes of 250 mM KPi, 2 M KBr for 48 h at 4 °C. Then the apoprotein was reconstituted by incubation in a 100-fold molar excess of FAD at room temperature overnight. Thereafter the sample was dialysed 2 times against 3000 volumes of 10 mM NaPi, 10 mM NaCl, pH 8.0, at 4 °C to remove excess FAD.

For chromophore analysis $Slr1694_I$ and $Slr1694_{II}$ were treated in the following way: A concentrated protein sample was denatured at 80 °C for 2 min and cooled on ice immediately afterwards. The precipitate was removed at 11,000*g* for 1 min in a bench-top centrifuge. The supernatant was filtered through a 0.45 μm syringe filter (Millipore) and applied to a reversed phase C18 column (250×4.6 mm, 4 μm pore-size, Prontosil, Bischoff Chromatographie, Leonberg Germany). The flavins were separated using 10 mM NH_4Ac (aq.) pH 6.0 (53 vol%) in acetonitrile (47 vol%) at a flow-rate of 0.8 ml/min. FAD, FMN and riboflavin, monitored by absorption at 365 nm, eluted after 20.5, 22.5 and 23.5 min, respectively. The ratio was calculated by the corresponding peak intensities.

2.2. Absorption spectroscopic characterization

The absorption cross-section spectra of fresh dark-adapted Slr1694 were determined by transmission measurements. The transmission spectra, $T(\lambda)$, were converted to absorption coefficient spectra, $\alpha(\lambda)$, by the relation $T(\lambda) = \exp[-\alpha(\lambda)\lambda]$, where λ is the sample path length. The absorption cross-section spectra, $\sigma_a(\lambda)$, were calculated from the absorption coefficient spectra, $\alpha(\lambda)$, by calibration to the absorption cross-section spectrum of FAD in aqueous solution at pH 7, i.e. the absorption cross-section integrals of Slr1694 and FAD extending over the S_0–S_1 and S_0–S_2 absorption band ($\lambda > 320$ nm) were set equal since the same isoalloxazine chromophore is present [38] [$\sigma_a(\tilde{\nu}) = \alpha(\tilde{\nu}) \int \sigma_{a,FAD}(\tilde{\nu})d\tilde{\nu} / \int \alpha(\tilde{\nu})d\tilde{\nu}$, where $\sigma_{a,FAD}(\lambda)$ is