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Absorption and fluorescence spectroscopic characterization of BLUF domain of AppA from *Rhodobacter sphaeroides*

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Abstract

The BLUF domain of the transcriptional anti-repressor protein AppA from the non-sulfur anoxyphototrophic purple bacterium *Rhodobacter sphaeroides* was characterized by absorption and emission spectroscopy. The BLUF domain constructs $AppA_{148}$ (consisting of amino-acid residues 1–148) and $AppA_{126}$ (amino-acid residues 1–126) are investigated. The cofactor of the investigated domains is found to consist of a mixture of the flavins riboflavin, FMN, and FAD. The dark-adapted domains exist in two different active receptor conformations (receptor states) with different sub-nanosecond fluorescence lifetimes ($BLUF_{r,f}$ and $BLUF_{r,sl}$) and a small non-interacting conformation ($BLUF_{nc}$). The active receptor conformations are transformed to putative signalling states ($BLUF_{s,f}$ and $BLUF_{s,sl}$) of low fluorescence efficiency and picosecond fluorescence lifetime by blue-light excitation (light-adapted domains). In the dark at room temperature both signalling states recover back to the initial receptor states with a time constant of about 17 min. A quantum yield of signalling state formation of about 25% was determined by intensity dependent transmission measurements. A photo-cycle scheme is presented including photo-induced charge transfer complex formation, charge recombination, and protein binding pocket reorganisation.

Keywords: BLUF domain; AppA; Blue-light photoreceptor; *Rhodobacter sphaeroides*; Absorption spectroscopy; Fluorescence spectroscopy; Fluorescence up-conversion; Photo-cycle; Flavoprotein; FAD; FMN; Riboflavin

1. Introduction

The blue-light response of biological organisms is an active field of research (for recent reviews see [1–10]). There are three classes of blue-light receptors, the cryptochromes [4,6,11,12] with sensing Cry domains, the phototropins Phot with LOV domains [5,13], and the regulatory proteins with BLUF domains [8,10,14–18]. 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.

BLUF domains (sensor for *b*lue *l*ight *u*sing *F*AD) are the light sensors of the multi-domain protein AppA from the purple non-sulfur anoxyphototrophic proteobacterium *Rhodobacter sphaeroides* [15], of PAC (photo-activated adenylylcyclase) from the unicellular flagellate *Euglena gracilis* [18], of Slr1694 from the cyanobacterium *Synechocystis* sp. PCC6803 [19], and of flavoproteins of at least 15 other microorganisms [8]. Blue-light excitation of BLUF domains leads to a slight red-shift of the absorption band and a recovery to the initial absorption behaviour in the dark [15,16,18–22].

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The flavoprotein AppA [8,14–17] from the non-sulfur anoxyphototropic purple bacterium *R. sphaeroides* controls photosynthetic gene expression in response to blue light exposure as well as in response to changes in the cellular redox state by functioning as an anti-repressor of the photosynthesis repressor protein PpsR [8,16,17,19,23]. Blue-light exposure of AppA in *R. sphaeroides* represses *puf* and *puc* operon expression in this bacterium [17,24]. AppA consists of an N-terminal BLUF domain and a cystein-rich C-terminal domain. The amino acid sequence of the BLUF domain of AppA is found in [8,15,20,21]. The photo-cycle dynamics of AppA by forming a long-living intermediate with slightly red-shifted absorption band was first described in [16]. In [20] a detailed spectroscopic and mutational analysis of the BLUF domain of $AppA_{1\text{-}156}$ (short: $AppA_{156}$, consisting of amino residues 1–156) was carried out including ultraviolet and visible spectroscopy, fluorescence measurements, nuclear magnetic resonance (NMR) spectroscopy, laser flash photolysis, and gel filtration chromatography. The photo-dynamics is interpreted in terms of flavin stacking with the amino acid Tyr-21 leading to fluorescence reduction [20]. It is proposed that light exposure strengthens a hydrogen bond between flavin and Tyr-21 leading to a stable local conformational change in $AppA_{1\text{-}156}$ [20]. In [21] the photo-cycle of $AppA_{5\text{-}125}$ was studied by UV–Vis spectroscopy, Fourier-transform infrared spectroscopy, pH measurements and site-directed mutagenesis. It is expected that $AppA_{5\text{-}125}$ in its dark-adapted state is protonated [N(5)-H], that it becomes exposed to solvent by blue-light exposure (light-adapted state, signalling state), and that an intra-molecular proton transfer from N(5) to anionic Tyr-21 forms the basis for the stabilisation of the signalling state [21]. Very recently – after finishing this paper – ultrafast time-resolved absorption and fluorescence spectroscopic studies on a femtosecond to nanosecond timescale and laser flash-photolysis studies on a nanosecond to microsecond timescale have been performed [25] to explore the photo-cycle dynamics of $AppA_{5\text{-}125}$. In the BLUF domain Slr1694 from *Synechocystis* sp. PCC6803 light-induced Fourier transform infrared (FTIR) spectroscopy indicated a weakening of the C(4)=O and C(2)=O bonding and a strengthening of the N(1)C(10a) and C(4a)N(5) bonding [19]. In investigating a recent heterologous expression of the BLUF domain from AppA [22] it was found that riboflavin, FMN, and FAD non-covalently bind to the domain and all three homologues have very similar blue-light photo-cycle dynamics.

In this paper the BLUF domains $AppA_{148}$ (domain consists of amino acid residues 1–148 with C-terminal His-tag) and $AppA_{126}$ (amino acid residues 1–126) from *R. sphaeroides* are characterized by absorption and emission spectroscopy. The flavin chromophore composition is analysed by thin-layer chromatography and fluorescence quantum yield measurement. Two active BLUF domain conformations and a third small conformation which does not form an intermediate are revealed by fluorescence lifetime and fluorescence quantum yield analysis. The fluorescence quantum yields and fluorescence lifetimes of the domains in the dark-state and in the light-adapted state are 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. All spectroscopic studies have been carried out at room temperature.

2. Experimental

A DNA-fragment encoding the BLUF protein AppA (Acc: L42555) from *R. sphaeroides* 2.4.1. was kindly provided by Dr. G. Klug (Giessen, Germany). Two fragments encoding amino acid 1–148 and 1–126, respectively, were amplified by PCR. AppA 1–126 was identified to be what one could call the protein's minimum BLUF domain by limited proteolysis of AppA 1–148 with Trypsin. Proteolytic fragments were analyzed by MALDI mass spectrometry and Edmann N-terminal sequencing. The two fragments were inserted between the NdeI and SacI restriction sites of a pET28a+ vector (Invitrogen, Karlsruhe), respectively. While the shorter construct only comprises an N-terminal His_6-tag, the longer construct possesses both, N- and C-terminal His_6-tag, rendering the purification procedure more effective. The C-terminal tag is attached to the AppA-coding region via the amino acid sequence AAPE and a short linker of vector-derived amino acid residues.

Escherichia coli cells (strain BL21) were transformed with these two constructs. Selected clones were grown in LB plus Kanamycine over night at 30 °C until the optical density at 600 nm had reached 0.5. The cultures were cooled down to 18 °C, and production of the AppA fragments was initiated by addition of 0.7 mM IPTG. Protein expression proceeded over night at 18 °C. The proteins were purified via Ni-NTA resin according to the instructions of the supplier (Quiagen, Hilden, Germany). They were subsequently exchanged in storage buffer (10 mM phosphate buffer, pH 8, 10 mM NaCl, 100 μM phenylmethylsulfonyl fluoride (PMSF)).

The flavin cofactors non-covalently bound to the AppA BLUF domains were determined by chromophore extraction from the protein, followed by thin-layer chromatography and fluorescence analysis. The chromophore was extracted from the protein following