Journal of Photochemistry and Photobiology B: Biology 101 (2010) 76–88

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Photophysical characterisation and photo-cycle dynamics of LOV1-His domain of phototropin from *Chlamydomonas reinhardtii* with roseoflavin monophosphate cofactor

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ARTICLE INFO

Article history:
Received 10 February 2010
Received in revised form 29 June 2010
Accepted 29 June 2010
Available online 3 July 2010

Keywords:
Roseoflavin monophosphate
Phototropin
Cofactor-exchanged LOV1 domain
RoLOV photo-cycle
LOV photo-cycle
Photo-induced protein re-conformation

ABSTRACT

The wild-type phototropin protein phot from the green alga *Chlamydomonas reinhardtii* with the blue-light photoreceptor domains LOV1 and LOV2 has flavin mononucleotide (FMN) as cofactor. For the LOV1-His domain from phot of *C. reinhardtii* studied here, the FMN chromophore was replaced by roseoflavin monophosphate (8-dimethylamino-8-demethyl-FMN, RoFMN) during heterologous expression in a riboflavin auxotropic *Escherichia coli* strain. An absorption and emission spectroscopic characterisation of the cofactor exchanged-LOV1-His (RoLOV1) domain was carried out in aqueous pH 8 phosphate buffer.

The fluorescence of RoLOV1 is quenched by photo-induced charge transfer at room temperature. The photo-cyclic dynamics of RoLOV1 was observed by blue-light induced hypochromic and bathochromic absorption changes which recover on a minute timescale in the dark. Photo-excited RoFMN is thought to cause reversible protein and cofactor structural changes. Prolonged intense blue-light exposure caused photo-degradation of RoFMN in RoLOV1 to fully reduced flavin and lumichrome derivatives. Photo-cycle schemes of RoLOV1 and LOV1 are presented, and the photo-degradation dynamics of RoLOV1 is discussed.

1. Introduction

Phototropins are blue-light sensitive photoreceptor proteins in plants [1–5]. They are involved in phototropism (plant growth towards light source), chloroplast movement, stomata opening, rapid inhibition of stem growth, and gametogenesis (sexual differentiation). In the unicellular green alga *Chlamydomonas reinhardtii* the phototropin receptor phot controls multiple steps in the sexual lifecycle of it [6,7]. The phot protein comprises two blue-light sensitive domains, LOV1 and LOV2 (LOV = light, oxygen, and voltage sensitive), in the N-terminal region and a serine-threonine protein kinase domain in the C-terminal region [8]. The LOV1 and LOV2 domains each non-covalently bind a flavin mononucleotide (FMN) molecule as cofactor for blue-light absorption and initiation of the photo-cyclic response. In the photo-cycle dynamics photo-excited FMN undergoes a FMN-C4a-cysteinyl adduct (signalling-state) formation with an adjacent cystein residue of the LOV domain protein which slowly recovers on a minute timescale back to the non-covalently bound receptor-state situation in the dark [9–11]. Blue-light induces global and localized conformational changes in the kinase domain of the full-length phototropin [12].

The pathway of FMN-C4a-cysteinyl adduct formation after photo-excitation of LOV domains was interpreted from experimental results (visible spectroscopy [13,14], crystal structure analysis [8,15,16], infrared spectroscopy [17], EPR spectroscopy [18,19], UV circular dichroism spectroscopy [20]) and was studied by quantum chemical methods on FMN–cystein models [21–23] and on complete LOV domain [24]. Different mechanisms of reaction of triplet excited flavin ^{3}FMN with adjacent Cys to FMN-C4a-cysteinyl adduct were proposed (ionic mechanism [13], concerted mechanism [8,15], sequential mechanism [14], radical pair mechanism [18,19,21–24]).

In riboflavin auxotroph strains (strains unable to biosynthesize riboflavin) of fungi (*Neurospora crassa* Rib2 strain [25,26], *Phycomyces* strain C222, *rib b3*(-) [27], *Trichoderma harzianum* Br183rib$^-$ strain [28]) FMN in the LOV domains could be replaced by roseoflavin (8-dimethylamino-8-demethyl-D-riboflavin) by *in vivo* growing in roseoflavin rich media, and the blue-light response of these modified fungi was investigated. Generally the photoreceptor action of the LOV domains with roseoflavin cofactor was found to be strongly reduced [25–28].

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doi:10.1016/j.jphotobiol.2010.06.014

Recently a method for generation of flavoproteins with modified cofactors *in vivo* was established [29]. Using this method flavins could be replaced by 8-amino-flavins during heterologous expression in riboflavin auxotrophic *Escherichia coli* strains. The cofactor exchanged flavoproteins have new biological, chemical, and physical properties. Earlier only amino acid mutations could be introduced and analysed to get insights into the flavoprotein function. Now the cofactor exchange extends the characterisation possibilities.

In a previous experiment the cofactor FAD (flavin adenine dinucleotide) of the BLUF protein Slr1694 from the cyanobacterium *Synechocystis* sp. PCC6803 was replaced by 8-dimethylamino-FAD (roseoflavin adenine dinucleotide) and the photo-biological behaviour was studied [30].

Here the LOV1 domain of phot from *C. reinhardtii* with the cofactor FMN (riboflavin 5′-monophoshate) replaced by 8-dimethylamino-8-demethyl-D-riboflavin 5′-monophosphate (roseoflavin monophosphate, RoFMN) is investigated. The LOV1 domain with RoFMN cofactor is abbreviated by RoLOV1. A short characterisation of RoLOV1 in [29] revealed a 15 nm blue shift of the first absorption maximum from 500 nm for free RoFMN in pH8 buffer solution to 485 nm and a characteristic fine structure for non-covalently bound RoFMN in RoLOV1. The fluorescence of RoFMN in RoLOV1 could not be extracted because of dominating fluorescence of some residual FMN or riboflavin (RF). No light-induced RoFMN-C4a-cysteinyl-adduct formation and no photo-cyclic behaviour were observed.

The 8-dimethylamino-flavins roseoflavin (8-dimethylamino-riboflavin, RoF) [31], roseoflavin monophosphate (roseoflavin mononucleotide, 8-dimethylamino-FMN, RoFMN) and roseoflavin adenine dinucleotide (8-dimethylamino-FAD, RoFAD) are the counterparts of riboflavin (RF) [32–35], flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Roseoflavin behaves bio-chemically and photo-chemically different than riboflavin: Riboflavin (vitamin B_2) is a cofactor in metabolism enzymes. Roseoflavin is an antibiotic against some Gram-positive bacteria and is a riboflavin antagonist [36]. In riboflavin the fluorescence quantum yield is reasonably high and limited by photo-physical internal conversion and intersystem crossing [37]. In roseoflavin the fluorescence quantum yield is very low because of photo-induced intra-molecular charge transfer and subsequent charge recombination [38].

In this paper the absorption and emission spectroscopic behaviour of RoLOV1 in aqueous pH 8 phosphate buffer (10 mM NaH_2PO_4/Na_2HPO_4 and 10 mM NaCl; abbreviated by NaP_i) is studied in some detail. The absorption cross-section spectrum of RoFMN in RoLOV1 is determined. The RoFMN cofactor loading in RoLOV1 is estimated. The thermal stability of the RoLOV1 domain is investigated. Fluorescence quantum distributions and fluorescence quantum yields are extracted from fluorescence spectra measurements. Fluorescence decay traces were measured. The fluorescence behaviour of RoFMN in RoLOV1 dissolved in pH 8 NaP_i buffer is discussed in terms of photo-induced intra-molecular charge transfer [39]. Light–dark photo-cyclic absorption and fluorescence behaviour was observed by blue-light exposure. The RoLOV1 putative receptor state–signalling state photo-cycle dynamics is discussed and the quantum efficiency of putative signalling-state formation is determined. Prolonged intense blue-light exposure caused photo-degradation of RoFMN in RoLOV1. The photo-degradation dynamics is studied and quantum yields of photo-degradation are determined.

The structural formulae of RoFMN and discussed flavin derivatives as well as the amino acid sequence of the RoLOV1 apo-protein are displayed in Fig. 1.

2. Experimental

RoLOV1 with His tag (more complete abbreviation RoLOV1-His) was prepared and analysed as previously described [29]. The samples were kept in pH 8 NaP_i buffer and stored at −80 °C. The measurements were performed at 4 °C except stated otherwise. Before measuring the thawed RoLOV1 sample debris was removed by centrifugation.

Transmission measurements were carried out with a commercial spectrophotometer (Cary 50 from Varian). Fluorescence emission and fluorescence excitation spectra measurements were carried out with a commercial spectrofluorimeter (Cary Eclipse from Varian).

Fluorescence lifetime measurements were performed with a mode-locked titanium-sapphire laser system (Hurricane from Spectra Physics) and an ultrafast streak-camera (type C1587 temporal disperser with M1952 high-speed streak unit from Hamamatsu) [38]. Picosecond excitation pulses at 456 nm were generated by stimulated Raman scattering of second harmonic pulses of the Ti:sapphire laser in ethanol (Stokes shift 2928 cm^{-1}) [40] using a Raman generator– system [41] with two 5 cm cells in series (focusing of 402 nm laser pulses to cells with a 50 cm lens, distance between cells ca. 5 cm for Raman light divergence reduction).

Photo-cycle studies were carried out by sample excitation with a high-pressure mercury lamp in combination with interference filters. Absorption spectra, fluorescence excitation spectra, and fluorescence emission spectra were measured at certain times during exposure and at certain times after excitation light switch-off.

The photo-degradation of non-covalently bound RoFMN ($RoFMN_b$) in RoLOV1 was studied by long-time sample excitation with a high-pressure mercury lamp in combination with band-pass filters (experimental setup is described in [42]). The photo-degradation of released free RoFMN ($RoFMN_f$) was studied by mercury lamp excitation at 546 nm (10 nm FWHM).

3. Results

3.1. Photo-physical behaviour

The cofactor composition of RoLOV1 was determined by reverse phase HPLC analysis in [29]. Only RoFMN and traces of FMN and riboflavin (RF) were found to be present. In a fluorescence spectra analysis we found the presence of a fraction of about 8.5% of FMN or RF of the total flavin content (see below). This fraction of FMN or RF could be identified to be dominantly free in solution from the spectral shape and spectral position: non-covalently bound FMN has a blue-shifted and vibronic structured emission spectrum compared to the free flavin [9].

The cofactor loading of the protein was determined by comparison of the absorbance of the holo-protein and the cofactor, which was released by heat denaturation and separated from the apo-protein by centrifugation [29]. The absorbance spectra, $A(\lambda) = -\log[T(\lambda)]$, of the holo-protein before denaturation and of the supernatant after denaturation are shown in Fig. 2a. A comparison of the absorbance of the supernatant with the absorbance of the holo-protein (1 Trp and 4 Tyr present) at 280 nm gives a RoFMN, FMN and RF cofactor loading factor of $\kappa_{load} = (N_{FMN+RF} + N_{RoFMN})/N_{apoprotein} = 0.28 \pm 0.03$, and a RoFMN cofactor loading of $\kappa_{RoFMN} = N_{RoFMN}/N_{apoprotein} = 0.26 \pm 0.03$ (N_i is number density of component i, for calculation procedure see for example [42]). The absorption cross-sections of $\sigma_{a,Trp}(280\ nm) = 2.02 \times 10^{-17}\ cm^2$ [43], $\sigma_{a,Tyr}(280\ nm) = 4.63 \times 10^{-18}\ cm^2$ [43], $\sigma_{a,FMN}(280\ nm) = 8.47 \times 10^{-17}\ cm^2$ [9], and $\sigma_{a,RoFMN}(280\ nm) = 6.32 \times 10^{-17}\ cm^2$ [30] were used in this analysis.

The thermal stability of RoLOV1 was studied by measuring the absorption spectrum of RoLOV1 as a function of time at a fixed temperature. An example is shown in Fig. 3 for a sample that was stored for 3 months at −20 °C and then investigated at room temperature (22 °C). At the start of measurement the sample