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Absorption and fluorescence spectroscopic characterization of cryptochrome 3 from *Arabidopsis thaliana*

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Abstract

The blue light photoreceptor cryptochrome 3 (cry3) from *Arabidopsis thaliana* was characterized at room temperature in vitro in aqueous solution by optical absorption and emission spectroscopic studies. The protein non-covalently binds the chromophores flavin adenine dinucleotide (FAD) and *N*5,*N*10-methenyl-5,6,7,8-tetrahydrofolate (MTHF). In the dark-adapted state of cry3, the bound FAD is present in the oxidized form (FAD_{ox}, ca. 38.5%), in the semiquinone form ($FADH^{\cdot}$, ca. 5%), and in the fully reduced neutral form ($FAD_{red}H_2$) or fully reduced anionic form ($FAD_{red}H^-$, ca. 55%). Some amount of FAD (ca. 1.5%) in the oxidized state remains unbound probably caused by chromophore release and/or denaturation. Förster-type energy transfer from MTHF to FAD_{ox} is observed. Photo-excitation reversibly modifies the protein conformation causing a slight rise of the MTHF absorption strength and an increase of the MTHF fluorescence efficiency (efficient protein conformation photo-cycle). Additionally there occurs reversible reduction of bound FAD_{ox} to $FAD_{red}H_2$ (or $FAD_{red}H^-$, FAD_{ox} photo-cycle of moderate efficiency), reversible reduction of $FADH^{\cdot}$ to $FAD_{red}H_2$ (or $FAD_{red}H^-$, $FADH^{\cdot}$ photo-cycle of high efficiency), and modification of re-oxidable $FAD_{red}H_2$ (or $FAD_{red}H^-$) to permanent $FAD_{red}H_2$ (or $FAD_{red}H^-$) with low quantum efficiency. Photo-excitation of MTHF causes the reversible formation of a MTHF species (MTHF′, MTHF photo-cycle, moderate quantum efficiency) with slow recovery to the initial dark state, and also the formation of an irreversible photoproduct (MTHF″).

Keywords: Cryptochrome; *Arabidopsis thaliana*; Cry3; MTHF; FAD; Fluorescence spectroscopy; Absorption spectroscopy; MTHF–FAD interaction; Photo-reduction; Photo-cycle dynamics; Energy transfer

1. Introduction

Blue-light sensitive photoreceptors control many crucial biological processes (for reviews see [1,2]). Important blue-light receptor groups are (i) cryptochromes (Cry) [3–5] that are related to photolyases, and which regulate plant growth and development as well as the synchronization of the circadian rhythm in animals, (ii) BLUF domain receptors [6] for the control of photosynthetic gene expression (AppA from *Rhodobacter sphaeroides* [7]), for blue-light avoidance response (PAC from *Euglena gracilis* [8]), for probably positive phototaxis (Slr1694 from *Synechochocystis* sp. PCC6803 [9]), and for still unknown function (Tll0078 from *Thermosynechococcus elongates* BP-1 [10], BrlB from *Rhodobacter sphaeroides* [11,12]), and (iii) phototropins (phot) [13] for the regulation of phototropism and other movement responses in plants. The chromophores (cofactors) in photolyases are flavin-adenine-dinucleotide (FAD) together with either (*N*5,*N*10)-methenyl-5,6,7,8-tetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deaza-riboflavin (8-HDF), while in cryptochromes FAD and MTHF were identified as chromophores [14,15]. In

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the BLUF domain receptors the cofactor is FAD, and in the phototropins the chromophore is flavin-mono-nucleotide (FMN).

Cryptochromes are UV-A and blue-light photoreceptors in plants, animals, and bacteria with a high degree of sequence and structural homology to DNA photolyases but with no repair function [4]. In the plant *Arabidopsis thaliana* three cryptochromes, cry1 [16], cry2 [17], and cry3 [18] (the latter also called cry-DASH [19]) have been identified. Cry1 plays a major role in the de-etiolation response [20]. Cry2 is involved in the day-length perception for flower induction [21], whereas the biological function of cry3 is not yet known.

In this paper, we undertook an optical spectroscopic characterization of cry3 from *Arabidopsis thaliana*. Cry3 was studied in vitro in an aqueous buffer solution at room temperature. Some comparative studies on MTHF-Cl were carried out. The dark-state absorption and emission behaviour of cry3 was investigated, followed by analysis of the absorption and emission behaviour under light excitation in the visible and UV-A spectral range. The behaviour of the sample after light exposure in the dark was also analyzed. The specific effects due to the presence of two chromophores, FAD (non-covalently bound FAD_{ox}, $FADH^{\cdot}$, $FAD_{red}H_2$, small fraction of unbound FAD_{ox}) and MTHF, are elucidated. Structural formulae of these molecules are depicted in Fig. 1.

2. Experimental procedures

2.1. Sample preparation

Cloning, expression in *Escherichia coli* and chromatographic purification of cry3 has been described elsewhere [22]. In brief, cry3 was overexpressed in *E. coli* M15[pREP4] cells that were grown after IPTG (isopropyl-β-D-thiogalactoside) induction in 1.5 l of LB medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin at 30 °C for 12 h by shaking at 250 rpm. The overexpressed cry3 protein was then purified by the following chromatography steps on the ÄKTApurifier (Amersham Biosciences, Buckinghamshire, UK): By Ni^{2+}-affinity chromatography on a HisTrap HP column, by Heparin chromatography on HiTrap Heparin HP column, and finally by size-exclusion chromatography on Superdex 200 GL column in buffer containing 50 mM Na-phosphate pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol and 10% glycerol. The final concentration of NaCl in the sample was adjusted to 50 mM, the other components remained unchanged (this final composition of the buffer is abbreviated as pH 7.5 buffer). Purified cry3 was concentrated to about 3–10 mg ml^{-1} as estimated by the Bradford method and analyzed by SDS–PAGE under reducing conditions. The identity of cry3 was confirmed by MALDI-TOF MS analysis (data not shown). The sample was divided into several aliquots, flash-frozen in liquid nitrogen and stored at −80 °C or

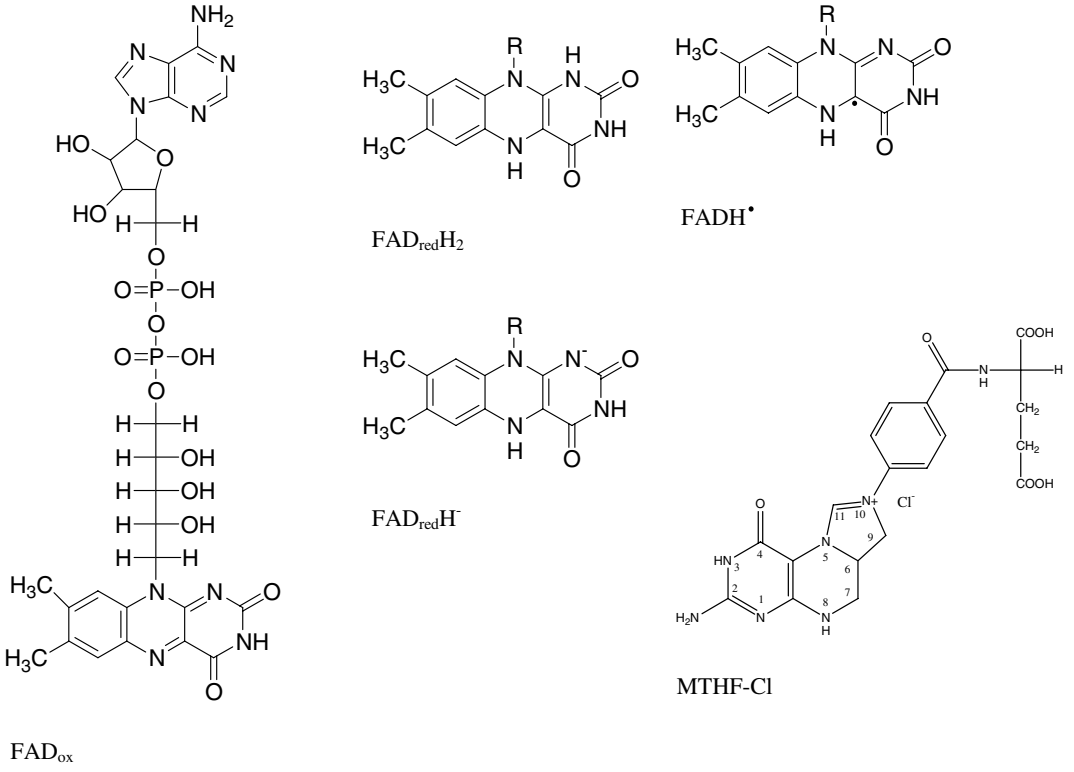

Fig. 1. Structural formulae of FAD in its oxidized form (FAD_{ox}), fully reduced neutral form ($FAD_{red}H_2$), fully reduced anionic form ($FAD_{red}H^-$), neutral semiquinone form ($FADH^{\cdot}$), and of MTHF-Cl.