CryB from *Rhodobacter sphaeroides*: a unique class of cryptochromes with new cofactors

Yann Geisselbrecht¹, Sebastian Frühwirth², Claudia Schroeder¹, Antonio J. Pierik³, Gabriele Klug²
& Lars-Oliver Essen^{1*}

¹Department of Chemistry—Institute of Biochemistry, Philipps-University, Marburg, ²Department of Biology and Chemistry—Institute of Microbiology and Molecular Biology, Justus-Liebig-University, Giessen and ³Department of Medicine—Core Facility for Protein Spectroscopy, Institute of Cytobiology and Cytopathology, Philipps-University, Marburg, Germany

Cryptochromes and photolyases are structurally related but have different biological functions in signalling and DNA repair. Proteobacteria and cyanobacteria harbour a new class of cryptochromes, called CryPro. We have solved the 2.7 Å structure of one of its members, cryptochrome B from *Rhodobacter sphaeroides*, which is a regulator of photosynthesis gene expression. The structure reveals that, in addition to the photolyase-like fold, CryB contains two cofactors only conserved in the CryPro subfamily: 6,7-dimethyl-8-ribityl-lumazine in the antenna-binding domain and a [4Fe-4S] cluster within the catalytic domain. The latter closely resembles the iron–sulphur cluster harbouring the large primase subunit PriL, indicating that PriL is evolutionarily related to the CryPro class of cryptochromes.

Keywords: CryPro; cryptochrome; iron–sulphur cluster; lumazine-binding protein; *Rhodobacter sphaeroides*

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INTRODUCTION

Photolyases and cryptochromes form a divergent enzyme family occurring in all three kingdoms of life, most likely because of the need on early forms of life on Earth to counter DNA damage caused by ultraviolet-B radiation. Accordingly, photolyases are efficient enzymes repairing ultraviolet lesions of DNA, namely the cyclobutane pyrimidine dimer (CPD) or the (6-4) pyrimidine–pyrimidone photoproduct ((6-4) photoproduct) [1]. In contrast, cryptochromes are involved in biochemical responses of organisms towards blue/ultraviolet-A light, for example, the circadian clock or phototaxis of plants. Until now, cryptochromes were supposed to have arisen either from class I CPD photolyases such

as plant and DASH-type cryptochromes or from (6-4) photolyases such as animal cryptochromes [2]. The discrimination between photolyases and cryptochromes became recently blurred as cryptochromes, such as the DASH-type or CryA from *Aspergillus nidulans*, exert dual functions by being competent in signalling and DNA repair [3,4].

One hallmark of photolyases and cryptochromes is their bilobal architecture featuring an amino (N)-terminal Rossmann fold and a carboxy (C)-terminal catalytic domain. The catalytic cofactor flavin adenine dinucleotide (FAD) is bound in an U-shaped conformation, adopts in photolyases all three possible oxidation states [2] and is indispensable for catalytic or signalling processes [1]. The N terminus might bind antenna chromophores to enhance absorbance cross-sections towards blue light [5]. Recently, structural homology between the catalytic domain of *Arabidopsis thaliana* cryptochrome 3 and the Fe/S-cluster sub-domain of the archaeal/eukaryotic primase subunit PriL [6,7] was shown, suggesting a plausible evolutionary relationship.

Cryptochrome B from *R. sphaeroides* (RsCryB) is a new member of the photolyase/cryptochrome family that controls the expression of genes of the photosynthetic apparatus. RsCryB transcription itself is under control of the RpoH_{II} sigma factor, which has a major role in response to singlet oxygen [8], indicating a possible link between light flux and stress caused by reactive oxygen species. Although RsCryB uses FAD as chromophore and undergoes photoreduction similar to other photolyases or cryptochromes [9], it is structurally only distantly related to them. Here, the crystal structure of cryptochrome B from *R. sphaeroides* is reported at 2.7 Å resolution. As the first structurally characterized cryptochrome of the proteobacterial (CryPro) class, RsCryB shows that CryPro-type cryptochromes use a hitherto unknown set of cofactors.

RESULTS AND DISCUSSION

RsCryB is a member of the CryPro subfamily

A database search starting from RsCryB (Uniprot entry Q3IXP1) yields a large, phylogenetically distinct cluster within the photolyase/cryptochrome family that is represented by INTERPRO

¹Department of Chemistry—Institute of Biochemistry, Philipps-University, Marburg, ²Department of Biology and Chemistry—Institute of Microbiology and Molecular Biology, Justus-Liebig-University, Giessen,

³Department of Medicine—Core Facility for Protein Spectroscopy, Institute of Cytobiology and Cytopathology, Philipps-University, Marburg, Germany

*Corresponding author. Tel: +49 6421 28 22032; Fax: +49 6421 28 22191; E-mail: essen@chemie.uni-marburg.de

family IPR007357 and Pfam-families DPRP PF04244 (desoxyribopyrimidine photolyase-related protein; supplementary Fig S1 online) and PF03441 (FAD-binding type 7 domain). Members of this cluster are highly abundant in proteobacteria and cyanobacteria. Therefore, we define this cluster as the new subfamily of CryPro. A multiple sequence alignment of the CryPro subfamily (supplementary information online) shows a signature of four conserved cysteines at the C-terminal catalytic domain.

A [4Fe-4S] cluster within RsCryB

Biochemical analyses indicated one [4Fe-4S] cluster per RsCryB molecule, because iron bound to recombinant RsCryB in a 3.7:1 ratio and acid-labile sulphur in a 3.9:1 ratio. Electron paramagnetic resonance (EPR) spectra of reduced (4 mM dithionite), oxidized (2 mM $K_3[Fe(CN)_6]$) and untreated RsCryB were recorded to reveal the nature of the cluster (supplementary Fig S2 online). Only in the oxidized sample an EPR signal characteristic for spin-coupled paramagnetic ions was detected, which broadened above 40 K and had g -values of 2.060 and 2.005. The signal was substoichiometric (~ 0.2 spin/RsCryB), suggesting that either the redox midpoint potential is higher than that of the ferri-/ferrocyanide redox couple or that the signal derives from an oxidative breakdown product of the [4Fe-4S] cluster. A bacterial ferredoxin-like [4Fe-4S] $^{1+/2+}$ cluster is unlikely in RsCryB because of the prominent signal in its oxidized form and the absence of a signal upon reduction. Nevertheless, the occurrence of an EPR signal upon oxidation makes it likely that native RsCryB contains a [4Fe-4S] cluster that converts to a structure with paramagnetic properties ($S=1/2$, $g_{average}>2$) similar to [3Fe-4S] $^{1+}$ or [4Fe-4S] $^{3+}$ clusters occurring in aconitase and high-potential Fe/S proteins, respectively. Such findings have been also reported for AddAB helicase [10], human and yeast primases, which have a [4Fe-4S] $^{2+}$ cluster in the native protein and contain EPR signals upon oxidation unlike other well-characterized Fe/S clusters [11].

The X-ray structure of RsCryB

The crystal structure of RsCryB was solved at 2.7 Å resolution by single-wavelength anomalous dispersion (SAD) techniques using the anomalous scattering of the bound [4Fe-4S] cluster (Table 1). RsCryB shows an N-terminal domain consisting of five helices plus a parallel, five-stranded β -sheet and a C-terminal, FAD-binding domain consisting of 18 α -helices (Fig 1A). A structural alignment between RsCryB and the structures of *Escherichia coli* CPDI photolyase [12], *Methanosarcina mazei* CPDII photolyase [13], *Drosophila melanogaster* (6-4) photolyase [14], *A. thaliana* Cry1 [15] and *A. thaliana* Cry3 [16] shows significant differences, especially in the catalytic FAD-binding region, whereas residues otherwise highly conserved in the photolyase/cryptochrome family are replaced (Fig 2A). In addition, the C-terminal end of RsCryB forms a unique, roof-like subdomain (S424–V509; Figs 1B,2B,C). RsCryB's linker is very long (amino acids 124–226, Fig 1A) and wraps partly around the catalytic domain, forming an additional wall of the canyon-like groove around the active site, which commonly accommodates DNA substrate duplexes in photolyases (Fig 3A,B). This modified groove allows nonspecific DNA binding with high affinity as demonstrated before [9].

Table 1 | Statistics for Data collection, processing and refinement

Data collection and processing	RsCryB
X-ray source	ESRF, Grenoble, France
Detector	ADSC Quantum Q315r
Wavelength (Å)	1.319
Space group	$P6_522$
Cell dimensions (a, b, c Å/ α, β, γ °)	137.8, 137.8, 521.9 90.0, 90.0, 120.0
Resolution (Å)	25.0–2.7
Total reflections	1,239,850
Multiplicity	15.2 (15.2)
Unique reflections	81,535
R_{merge} (%)	0.100 (0.563)
Completeness (%)	99.8 (99.8)
$I/\sigma(I)$	26.9 (5.4)
Wilson B-factor (Å ²)	48.5
Refinement statistics	
Resolution (Å)	25.0–2.7 (2.77–2.70)
R_{factor} , R_{free} (%)	19.9, 22.8
Reflections (working, test set)	79,535 (2,000)
Completeness for range (%)	100.0
r.m.s.d. from ideal:	
Bond lengths (Å)	0.016
Bond angles (°)	1.55
Total number of atoms	12,516
Mean B value (Å ²)	32.2

ESRF, European Synchrotron Radiation Facility; r.m.s.d., root mean square deviation; RsCryB, cryptochrome B from *R. sphaeroides*.

The [4Fe-4S] cluster is part of the roof-like subdomain

The cluster is enclosed between the roof-like subdomain and the catalytic domain and clearly defined by electron density as surface-occluded [4Fe-4S] cluster. The cluster is at a distance of 22.9 Å to FAD (Fig 4C), making direct electron transfer between both cofactors, for example, during photoreduction, rather unlikely [17]. RsCryB's [4Fe-4S] cluster is coordinated by four cysteines conserved within the CryPro family (C346, C434, C437 and C450, Fig 4C). Five hydrogen bonds to sulphide and cysteine ligands (supplementary Fig S3 online) indicate that the cubane resembles high-potential Fe/S proteins more than it does bacterial ferredoxins [18,19], in agreement with EPR data and the lack of solvent access. Nevertheless, the [4Fe-4S] cluster is adjacent to the putative DNA-binding region of RsCryB (Fig 3B) and might be hence affected by DNA binding. Interestingly, the PriL-CTD subunits of eukaryotic primases structurally align not only with RsCryB in the catalytic domain, but also bear their [4Fe-4S] cluster at the same end (Fig 4D). This structural relationship of RsCryB to a DNA-binding primase domain, as well as the conservation of

Fig 1 | The X-ray structure of RsCryB. (A) Architecture of RsCryB with the N-terminal antenna-binding domain (blue) and a long linker region (salmon) wrapping the catalytic domain (orange). (B) Overlay of RsCryB (3ZXS, blue, thick lining) with EcCPDI (1DNP, turquoise, 2.9 Å), MmCPDI (2XRY, brown, 2.6 Å), Dm(6-4) (3CVU, magenta, 3.1 Å), AtCry3 (2J4D, yellow, 3.0 Å) and AtCry1 (1U3C, green, 3.0 Å). In parenthesis is given the PDB accession code, the colour in the figure and the root mean square deviation of C α 's between this structure and RsCryB. The coloured boxes refer to the antenna-binding domain (blue), the core of the catalytic domain (orange) and the roof-like subdomain of RsCryB, harbouring the [4Fe-4S] cluster (green). C-term, carboxy terminus; CPD, cyclobutane pyrimidine dimer; DLZ, 6,7-dimethyl-8-ribityl-lumazine; FAD, flavin adenine dinucleotide; N-term, amino terminus; PDB, protein data bank; RsCryB, cryptochrome B from *R. sphaeroides*.

crucial residues such as the first cysteine for coordinating the [4Fe-4S] cluster (RsCryB: C346; ScPriL: C336) and surrounding R365 and F372 (ScPriL: R355, F361), hints to a direct evolutionary linkage between the CTD domain of ScPriL and the CryPro class of cryptochromes.

Binding site of the FAD chromophore

The FAD-binding pocket harbours the cofactor in the characteristic U-shaped conformation, but shows several features hitherto unobserved [12,14–16,20,21]. First, a single water molecule (w1) is suitably placed to form H bonds both to the N5 nitrogen of FAD and the backbone carbonyl of Y387 (Fig 2A). This clearly differs from all other photolyases and cryptochromes where the cofactor interacts either with an asparagine (EcCPDI: N378 [12], MmCPDI: N403 [13], Dm6-4: N403 [14], and AtCry3: N428 [16]) or aspartate (AtCry1: D396 [15]). Asparagine allows FAD to undergo complete photoreduction/protonation (FAD_{ox} → FADH → FADH[−]), whereas aspartate arrests the radical FADH state. In RsCryB, which photoreduces to FADH[−] [9], the corresponding residue, E399, swivels away from the N5 nitrogen, forming a salt bridge with H384.

Second, the side chain of Y387 replaces a conserved tryptophan next to the FAD that is, in other enzymes, essential for photoreduction as part of a tryptophan triad (EcCPDI: W381 [12], Dm6-4: W407 [14], AtCry3: W432 [16], AtCry1: W400 [15]; Fig 2A–C). In RsCryB, mutagenesis analysis of the putative electron transfer pathway shows that at least two tryptophans, W338 and W386, are crucial (supplementary Fig S4 online), as mutagenesis of each tryptophan to phenylalanine abolished photoreduction. These residues are characteristic for CryPro cryptochromes, but structurally distinct from the canonical tryptophan triad. Surprisingly, replacement of Y387, or of an alternative aromatic residue proximal to FAD and close to W386 (Y391), by phenylalanine fails to ablate photoreduction (supplementary Fig S4 online), with activities of 77% and 37% of the wild-type protein. Similarly, a double mutant was only partly

impaired in the overall photoreduction with a relative rate of 49%. These data indicate a mode of direct electron transfer between W386 and FAD. Interestingly, class II photolyases also use a tryptophan dyad as pathway for photoreduction, but this is again different from that of CryPro and other cryptochrome classes [13]. Finally, further replacements of conserved amino acids close to the adenine moiety of FAD set this representative of the CryPro subfamily clearly apart from other photolyases and cryptochromes.

In RsCryB, the strongly basic groove around the FAD-binding site shows similar electrostatic charge distributions at the DNA-binding grooves of other photolyases (Fig 3A). However, this highly conserved groove (supplementary Fig S5 online) is sterically constricted by the linker after helix- α 7, corroborating the findings that RsCryB binds single-stranded DNA ($K_D \sim 10^{-8}$ M) better than double-stranded DNA ($K_D \sim 10^{-6}$ M) and neither repairs CPD lesions [9] nor (6-4) photoproducts (supplementary Fig S2 online).

The antenna chromophore of RsCryB

RsCryB binds an additional chromophore ($\lambda_{\text{max}} = 420$ nm), which evaded identification earlier [9]. The N-terminal domain now shows well-defined electron density that is too small for known antenna chromophores such as MTHF, FMN, FAD or 8-HDF [1,22,23], but clearly matched with DLZ (Fig 4A,B). DLZ, the last intermediate of the riboflavin biosynthesis pathway, is a plausible chromophore for RsCryB, as it acts as an accessory fluorescent chromophore in luciferases of many luminescent marine bacteria [24,25]. Mass-spectrometric analyses of cofactors released from recombinant RsCryB yielded a mass of 327.1293 Da, which corresponds to the calculated mass of protonated DLZ (C₁₃H₁₉N₄O₆: 327.1299 Da; supplementary Fig S2 online).

The DLZ chromophore forms numerous interactions within its binding pocket that is unique for the CryPro subfamily as shown by multiple sequence alignment (supplementary information

Fig 2 | Analysis of the FAD-binding site and phylogenetic relationships of RsCryB. (A) FAD-binding site of RsCryB. The central water W1 hydrogen bonds to the N5 of the isoalloxazine ring. (B) Structural alignment between RsCryB and other photolyases/cryptochromes. The canonical tryptophan triad (violet) conserved within the latter is not found in RsCryB. The CryPro-specific cysteines are highlighted in red. (C) RsCryB with its proteobacterial and cyanobacterial relatives forms a distinct CryPro cluster inside the photolyase/cryptochrome family. CPD, cyclobutane pyrimidine dimer; CryPro, proteobacterial cryptochromes; FAD, flavin adenine dinucleotide; RsCryB, cryptochrome B from *R. sphaeroides*.

online). Its aromatic ring is sandwiched between E37, I51, M55 and I83. A32 together with V34 has a pivotal role by forming hydrogen bonds to the imide substructure of the pterin ring. V34 additionally positions one water molecule for coordination of the N5 nitrogen. Furthermore, the ribityl group of DLZ forms hydrogen bonds to D10, E37 and Y40. The two methyl groups

are positioned in a hydrophobic pocket mainly built up by I48, I50 and K47 (Fig 4A,B), which at the same time block binding of the benzo moiety of flavin-like antenna chromophores. The DLZ chromophore is 18.8 Å away from the FAD, a distance comparable to that of other antenna chromophores (*TrCPDI*/FMN: 17.2 Å; *AnCPDI*/8-HDF: 17.5 Å). DLZ can be therefore expected to excite

Fig 3 | Analysis of electrostatic surface potentials as calculated by APBS software [32]. (A) All members of the photolyase/cryptochrome family show a basic groove around a hole, granting access for DNA lesions towards the catalytic FAD. (B) In RsCryB, a putative, more restricted groove suitable for accommodating at least single-stranded nucleic acids is indicated. FAD, flavin adenine dinucleotide; RsCryB, cryptochrome B from *R. sphaeroides*.

the FAD via resonant energy transfer, similar to other antenna chromophores of the nucleotide type.

The utilization of 6,7-dimethyl-8-ribityl-lumazine as antenna illustrates the promiscuity with which photolyases and cryptochromes recruit chromophores for broadening the absorbance cross-section of their catalytic FAD cofactor. DLZ is a highly efficient antenna chromophore, when compared with riboflavin-like chromophores, as it has a higher quantum yield for fluorescence (0.6 versus 0.38) and similar extinction coefficients (10.3 versus 12.5 mM⁻¹ cm⁻¹). Accordingly, in marine organisms from the genus *Photobacteria*, DLZ-binding proteins are known to boost bacterial bioluminescence by forming stoichiometric complexes with luciferases [24].

CONCLUSION

RsCryB provides the first structure of the herein defined CryPro subfamily of cryptochromes with the [4Fe-4S] cubane as most prominent part of the C-terminal domain. Although the function of this Fe/S-cluster remains to be explained, *cryB* transcription of *R. sphaeroides* increases in response to singlet oxygen and RsCryB itself affects the regulation of photosynthesis-related genes [8,9]. EPR spectroscopy showed that the [4Fe-4S] cluster of RsCryB can readily be oxidized, and thus RsCryB might act as sensor for reactive oxygen species as a result of photooxidative stress. Oxidative damage of the [4Fe-4S] cluster could trigger disordering or structural change of the C-terminal end and alter the nucleotide-binding, basic groove for either recognition or release of ligands such as certain RNA species [9]. Such a function of the [4Fe-4S] cluster is not without precedent, as Fe/S-clusters are intrinsic elements of many other DNA/RNA-binding enzymes including helicases, primases or RNA-dependent DNA polymerases [10], being made responsible for functions such as regulation, sensing or interaction with DNA. In this context, a

function of RsCryB as a cryptochrome instead of a DNA-repair enzyme is consistent with its lack of repair for CPD lesions or (6-4) photoproducts and the comparatively narrow surface groove around the catalytic site that allows only the binding of single-stranded nucleic acids (Fig 3A,B).

Overall, the CryPro subfamily described herein, of which another member with unknown biological function has been recently characterized [26], provides a new theme in the photolyase/cryptochrome family by presenting new ways of intramolecular selection and energy transfer.

METHODS

Crystallization of RsCryB. Recombinant RsCryB [9] was further purified by SEC on a Superdex 200 column in 10 mM Tris and 100 mM NaCl, pH 8.0. Initial crystallization attempts were made in 96-well sitting-drop Innovaplates (Jena Bioscience). The initial hit (0.3 M MgCl₂, 0.1 M bicine, pH 9.0, 25% PEG2000 and 15% glycerol) was further optimized in 24-well hanging-drop vapour diffusion setups and yielded 100 × 100 μm² platelets (0.3 M MgCl₂, 0.1 M bicine, pH 9.0, 29.5% PEG2000, 17.5% glycerol, and 7 mg/ml RsCryB). After soaking with 100 μM GdCl₃, the crystals were cryoprotected with 0.3 M MgCl₂, 0.1 M bicine, pH 9.0, 29.5% PEG2000 and 30% glycerol and flash-frozen in liquid nitrogen.

Structure determination. SAD data were recorded at 100 K using beamline ID14-4 of the European Synchrotron Radiation Facility, Grenoble, France. The long *c* axis of the hexagonal crystal form required fine slicing of recorded data (rotation about 0.06°) to avoid overlapping of reflexes. Data reduction with XDS and XSCALE [27] gave a data set with 2.7 Å resolution, a solvent content of 69% and three molecules per asymmetric symmetry unit (Table 1). SAD phasing was performed with AutoSol [28], and model building and refinement was carried out within PHENIX, REFMAC5 [29] of the CCP4 suite [30] and WinCoot [31]. All further analyses refer to molecule A

Fig 4 | The DLZ antenna and the [4Fe-4S]-cluster of RsCryB. (A) DLZ bound to the antenna-binding region. The ribityl moiety forms four distinct hydrogen bonds. The N5 nitrogen interacts with a water molecule (W2) held in place by a backbone carbonyl. Electron density in blue (SIGMA-weighted $2F_o - F_c$ map) is contoured at 1σ . (B) Scheme of the DLZ-binding site. (C) The [4Fe-4S] cluster is coordinated by four conserved cysteines. Helices $\alpha 19$, $\alpha 20$ and $\alpha 21$ form the roof-like subdomain. (D) Structural overlay (root mean square deviation C_α 's 2.6 Å) between a part of the yeast primase subunit PriL-CTD (3LGB, grey, L335-S422) and the core of the C-terminal domain of RsCryB (3ZXS, orange, $\alpha 14$ - $\alpha 18$, A345-R429). DLZ, 6,7-dimethyl-8-ribityl-lumazine; FAD, flavin adenine dinucleotide; RsCryB, cryptochrome B from *R. sphaeroides*.

if not otherwise stated. Centroid-centroid distances between aromatic moieties were calculated with EXCEL (Microsoft).

RsCryB purification for biochemical analysis. For improved expression, *cryB* was cloned into the pET28a vector and transformed into *E. coli* Rosetta(DE3) (Merck). After immobilized metal-ion affinity chromatography with a NiNTA-Sepharose column (GE Healthcare), a second purification step on a HiTrap heparin column (GE Healthcare) removed residually bound nucleic acids from RsCryB and yielded >95% pure RsCryB, which was assayed for iron and acid-labile sulphur and used in mass-spectrometric analysis and EPR experiments.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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