Interaction of two photoreceptors in the regulation of bacterial photosynthesis genes

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ABSTRACT

The expression of photosynthesis genes in the facultatively photosynthetic bacterium *Rhodobacter sphaeroides* is controlled by the oxygen tension and by light quantity. Two photoreceptor proteins, AppA and CryB, have been identified in the past, which are involved in this regulation. AppA senses light by its N-terminal BLUF domain, its C-terminal part binds heme and is redox-responsive. Through its interaction to the transcriptional repressor PpsR the AppA photoreceptor controls expression of photosynthesis genes. The cryptochrome-like protein CryB was shown to affect regulation of photosynthesis genes, but the underlying signal chain remained unknown. Here we show that CryB interacts with the C-terminal domain of AppA and modulates the binding of AppA to the transcriptional repressor PpsR in a light-dependent manner. Consequently, binding of the transcription factor PpsR to its DNA target is affected by CryB. In agreement with this, all genes of the PpsR regulon showed altered expression levels in a CryB deletion strain after blue-light illumination. These results elucidate for the first time how a bacterial cryptochrome affects gene expression.

INTRODUCTION

Bacteria have to respond to multiple external stimuli in order to guarantee survival in an environment with changing conditions. Many microorganisms are exposed to sunlight in their natural habitats. While light provides the energy for photosynthesis it is also harmful through the damaging effect of ultraviolet light and the generation of reactive oxygen species in presence of internal or external photosensitizers like chlorophyll, protoporphyrin or humic acids. Several microorganisms are able to respond to changes in light quantity, which is either directly sensed by photoreceptors or indirectly through the photosynthetic electron transport (1–3). Despite the growing number of photoreceptor proteins discovered in bacteria, up to date the biological function and the mechanisms of signaling are only understood for few of them.

Rhodobacter sphaeroides is a facultatively photosynthetic bacterium, found in fresh water habitats. At high oxygen tension it performs aerobic respiration and does not form photosynthetic complexes. If oxygen tension drops, genes for pigment synthesis and pigment binding proteins are induced and photosynthetic complexes are assembled. However, at intermediate oxygen levels light illumination leads to photosynthesis gene repression (4,5), most likely to avoid the generation of singlet oxygen. At low oxygen tension or anaerobic conditions formation of photosynthesis complexes is no longer repressed by light (1,4) and anoxygenic photosynthesis can be performed.

Rhodobacter sphaeroides harbors a set of different photoreceptors including two phytochromes, a LOV domain protein, three BLUF (Blue Light sensing Using FAD) domain proteins and a cryptochrome. Both phytochromes are composed of the PAS–GAF–PHY photosensory module, typically present in phytochromes, but linked to GGDEF–EAL output modules. One of the phytochromes, BphG1, was shown to be involved in the turn-over of c-di-GMP (6). The short LOV domain protein of *R. sphaeroides* lacks an output module and undergoes a photocycle but its biological function remains to be elucidated (7). Similarly, two of the BLUF domain proteins of *R. sphaeroides* lack an output domain (8) and their biological function is not known. The BLUF domain was first discovered in the AppA protein of *R. sphaeroides* (4,8,9), which was intensively studied in regard to its biological function, the mechanisms of signal transduction and its photocycle. The AppA protein was initially identified as a redox regulator of photosynthesis genes, which functions as antagonist of

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the PpsR protein (10,11). PpsR represses photosynthesis genes at high oxygen tension by binding to target promoters (11). Binding to PpsR is mediated by the C-terminal part of AppA (12), which was shown to bind heme (13,14). The novel type of heme-binding domain was named SCHIC (Sensor Containing Heme Instead of Cobalamin) domain (14). AppA also functions as photoreceptor through its BLUF domain, which interferes with PpsR binding at intermediate oxygen concentrations in response to blue light (4,9,12,13). Figure 1 shows a simplified schematic model for photosynthesis gene regulation by AppA/PpsR. Recently we demonstrated the involvement of the cryptochrome CryB in the regulation of photosynthesis genes in *R. sphaeroides* (15). The promoter of *cryB* is recognized by the RpoE-dependent alternative sigma factor $RpoH_{II}$, both sigma factors have a major role in the response of *R. sphaeroides* to photooxidative stress (16–19).

It remained however elusive, by which mechanisms CryB affects expression of photosynthesis genes. Since the affinity of CryB to double-stranded DNA was low (15), we considered that it may act on gene expression by interaction to other proteins.

Here we present results showing that CryB directly interacts with the C-terminal part of AppA. Gel shift experiments demonstrate that CryB interferes with the interaction of PpsR and AppA.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Supplementary Table S1. *Rhodobacter sphaeroides* strains were grown at 32°C in malate minimal salt medium. *Escherichia coli* was cultivated in Luria–Bertani broth at 37°C. *Rhodobacter* conjugation was performed as described elsewhere (20). When
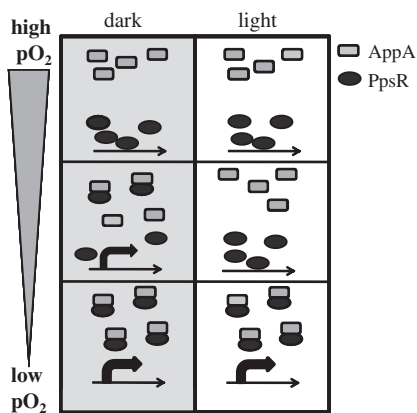
Figure 1. Simplified model of AppA/PpsR dependent gene regulation. Under high oxygen ($>4\,mg\,l^{-1}$ dissolved O_2) AppA (grey rectangle) is unable to bind PpsR (black circle) due to its oxidized heme and the free PpsR repressor inhibits photosynthesis gene expression. With decreasing oxygen levels AppA binds to PpsR thus photosynthesis gene expression is restored. At intermediate oxygen levels, however, illumination results in release of PpsR from AppA and consequently repression of photosynthesis genes (modified from 9). The arrows indicate transcription of photosynthesis genes.

required, antibiotics were applied in the following concentrations: kanamycin $25\,\mu g\,\mu l^{-1}$, ampicillin $200\,\mu g\,\mu l^{-1}$, tetracycline (*E. coli* $20\,\mu g\,\mu l^{-1}$, *R. sphaeroides* $2\,\mu g\,\mu l^{-1}$).

Genetic techniques

DNA isolation, restriction and cloning were performed according to standard protocols (21). Oligonucleotides for cloning were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Sequencing of cloned DNA fragments was performed with the ABI-Prism 310 genetic analyzer (Applied Biosystems, Carlsbad, USA).

Construction of GST-SCHIC overexpression

The 0.38 kb BamHI–EcoRI DNA fragment, containing the SCHIC domain codons 272–397, was amplified by PCR (using primers 5′-CAGGGATCCGTGGGCGCCG TGCTG-3′ and 5′-GGCGAATTCTCACACACGGGCG AGGGCG-3′) and cloned into the BamHI and KpnI sites of vector pGEX4T-1 (Amersham Biosciences, Freiburg, Germany). The recombinant plasmid designated pGEX*appA*SCHIC was transformed into *E. coli* JM109.

Protein overexpression and purification

His-CryB, GST-PpsR, MBP-AppA and GST-AppAΔN were overexpressed in *E. coli* JM109 (22) and purified as described previously (7,11,13,14). Overexpression of GST-SCHIC was induced in *E. coli* JM109(pGEX*appA*SCHIC) at 17°C overnight with 1 mM isopropyl-β-D-thiogalactopyranoside. The purification was performed using glutathione sepharose 4B according to the manufacturer's instruction (Amersham Biosciences, Freiburg, Germany). The eluted proteins were dialyzed in storage buffer (250 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 15% glycerol). Protein concentrations were quantified using the Bradford assay (23).

Analysis of interacting proteins by pull-down experiments

For analyzing interaction of the His-CryB (15) and His-LOV (7) protein with full-length maltose-binding protein (MBP)-AppA (14) or with GST-AppAΔN (13) the amylose resin (New England Biolabs, Frankfurt a. M., Germany))/the glutathione sepharose 4B (Amersham Biosciences, Freiburg) was equilibrated in PBSMT buffer (250 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 20 mM β-mercaptoethanol, 0.1% Trition X-100). The resin was then incubated with 50 μg purified and dialyzed MBP-AppA/GST-AppAΔN. After incubation for 1 h at room temperature the mixture was washed 15 times with PBSMT buffer. Afterwards, the protein-charged resin was incubated with cell extract (100 μg of total protein) from *R. sphaeroides* Δ*cryB*(pRK*pufcryB*) or *R. sphaeroides* 2.4.1(pRK*puflov*), respectively, and washed again 15 times with PBSMT buffer. Elution fractions were collected after adding MBP-elution buffer (250 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 50 mM maltose)/GST elution buffer (250 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM reduced glutathione). Western blots were performed using the Lumi-Light[PLUS] WesternBlotting Kit (Roche, Grenzach-Wyhlen, Germany) with rabbit antibodies against CryB (15) and LOV (7).

Electrophoretic mobility shift assay

GST-PpsR was preincubated with GST-AppAΔN and His-CryB at room temperature for 30 min and then incubated for further 30 min at room temperature with the ^{32}P-labeled *puc* DNA probe [30 fmol, −281 to −23 from start codon of the *pucB* gene containing two PpsR-binding sites (13)] in 10 µl of binding solution [50 mM Tris/HCl (pH 7.0), 1 mM EDTA, 150 mM NaCl, 10% glycerol and 1 µg of salmon sperm DNA]. Afterwards, the mixtures were subjected to 4% native polyacrylamide gel electrophoresis in Tris–acetate–EDTA buffer. The signals were analysed by using a phosphoimaging system (*Molecular Imager*® FX; Bio-Rad, Munich, Germany) and the respective imaging software (Quantity One; Bio-Rad, Munich). For illumination white light (intensity of blue light 2 µmol m^{-2} s^{-1}) was applied.

Construction of Two-Hybrid plasmids

Plasmids pGAD-T7 containing the sequence for the GAL4 activation domain and pGBK-T7 containing the gene for the binding domain of the GAL4 protein were used in the two-hybrid system (Matchmaker two-hybrid system 3; Clontech). The *cryB* gene was amplified from *R. sphaeroides* 2.4.1 chromosomal DNA with the oligonucelotides *cryB*pGBKT7NdeI*up* (3′- GGAATTC<u>CATATG</u>ACACG GCTCATCCTCGT-5′) and *cryB*pGBK<u>T7</u>EcoRI*down* (3′- <u>GGAATTC</u>GACCGGCTCGCCCGCGTG-5′), cut with the respective enzymes and cloned into pGBK-T7 resulting in plasmid pGBK-T7-*cryB*. For testing CryB interaction against different AppA domains these were cloned into the pGAD-T7 vector using the following oligonucleotides AppA: *appAfull*pGADT7NdeI*up* (3′-GGG<u>CATATG</u>ATG CAACACGACC-5′) and *appAfull*pGADT7SacI*down* (3′-<u>CGAGCTC</u>TCAGGCGCTGCGGCG-5′); AppAΔC: *app AΔC*pGAD<u>T7</u>NdeI*up* (3′-GG<u>CATATG</u>ATGCAACACG ACCTC-5′) and *appAΔC*pGAD<u>T7</u>SacI*down* (3′-CAT<u>GA GCTC</u>CCTCCGAGCAGGAG-5′); AppAΔN: *appAΔN*p GADT7NdeI*up* (3′-CAG<u>CATATG</u>ATGTCGGAGGCCG ACATG-5′) and *appAΔN*pGADT7SacI*down* (3′-<u>CGAGC TCT</u>CAGGCGCTGCGGCG-5′); AppAΔSCHIC: *appAΔ SHIC*pGADT7NdeI*up* (3′-GGG<u>CATATG</u>ATGCAACAC GACCTCG-5′) and *appAΔSHIC*pGADT7SacI*down* (3′- GAG<u>GAGCTC</u>TCAGAGGATCGGCTTG-5′); SCHIC: SHICpGADT7NdeI*up* (3′-CCG<u>CATATG</u>ATGGTGGGC GCCGTGC-5′) and SHICpGAD<u>T7</u>SacI*down* (3′-CATT<u>G AGCTC</u>TCAGGCCACACGGGC-5′).

Yeast methods

Yeast transformation was performed by applying the LiAc method (24) with a modified protocol (25). Clones containing only the plasmids pGAD-T7 and pGBK-T7 showed no β-galactosidase activity. After transformation cells were plated on solid SD medium lacking leucine, tryptophan and histidine, and incubated for 3–5 days at 30°C. The colonies were then replica plated on solid SD medium lacking leucine, tryptophan, histidine and adenine and incubated. Blue/white selection was performed by adding X-Gal (25 µg µl^{-1}, Carl Roth, Karlsruhe, Germany) to the SD plates (25,26). β-Galactosidase

activity of independent clones was quantified as described previously (27).

For following light-dependent interaction of CryB and the C-terminal part of AppA the yeast strain harboring pGBK-T7-*cryB* and pGAD-T7-*appAΔN* was grown in minimal medium over night. Next morning the culture was diluted to an OD$_{600}$ of ∼0.15 and grown for another 2 h. Then the culture was split and half of the culture was illuminated with blue light (20 µmol m^{-2} s^{-1}) the other half was kept in the dark. The cultures were kept at ∼90 µM dissolved oxygen.

Rhodobacter sphaeroides growth conditions and real-time RT–PCR

For blue light *in vivo* experiments, microaerobically grown (30 µM dissolved oxygen) *R. sphaeroides* cultures (wild-type or 2.4.1$\Delta cryB$) were diluted to an OD$_{660}$ of 0.15. Cultures were incubated under semiaerobic conditions (90 µM dissolved oxygen) by variation of the shaker speed. After one doubling time blue light (λ_{max} = 450 nm with 20 µmol m^{-2} s^{-1} on the culture level) was passed through a narrow band filter (4). Samples of three independent repeats were collected after 60 min blue light illumination and RNA was isolated for real-time RT–PCR using the peqGOLDTriFast Kit (peqlab). DNA was digested using DNase I (Invitrogen) and each sample checked for DNA contamination by PCR with wild-type DNA as positive control. RNA concentration was determined spectroscopically using the Nanodrop (Thermo Scientific) and a final concentration of 4 ng µl^{-1} total RNA was used for each real-time RT–PCR reaction. Following the specifications of the one-step RT–PCR kit (Qiagen) with the corresponding buffers and polymerases, duplicates of each real-time RT–PCR reaction were performed using the Rotor-Gene 300 ThermoCycler (Corbett Research). Non template controls without RNA added to the master mixture were used. Primers used for analyzing the expression of different target genes are listed with their corresponding efficiencies in Supplementary Table S2 of the Supplementary Data. Sybr green I (Sigma–Aldrich) was added in a final dilution of 1:50 000 to the master mixture. Crossing points (Cp) with a fluorescence threshold of 0.002 were visualized by the use of the Rotor-Gene software 6.0 (Corbett Research) and the relative expression of *cryB* mutant mRNAs was calculated relative to wild-type and the control gene *rpoZ* as described before (18,28).

RESULTS

Yeast two-hybrid analysis reveals an interaction between AppA and CryB

Our recent work demonstrated that the CryB protein of *R. sphaeroides* influences the expression of photosynthesis genes (15); however, the mechanism of this regulation was still elusive. To further elucidate the regulatory function of the CryB protein a yeast two-hybrid interaction analysis was performed against the AppA/PpsR system, which possesses a well understood function in the regulation of photosynthesis genes in *R. sphaeroides* (Figure 1). To this end the full-length *cryB* gene was cloned into the pGBK-T7

vector. The resulting vector pGBK-T7-*cryB* was then transformed into yeast competent cells together with a plasmid harboring the full-length *appA* (pGAD-T7-*appA*) gene. Colonies were first selected for growth on leucine/ tryptophan/histidine and later for a more stringent selection additionally for growth on adenine. Cotransformation with pGAD-T7-*appA* or with pGBK-T7-*cryB* led to growth on leucine/tryptophan/histidine/adenine (Figure 2) and positive blue/white selection. After the transfer of the plasmids pGBK-T7-*cryB* and pGAD-T7-*app*A into *Saccharomyces cervisiae* Y187 the strain showed ~40% of the β-galactosidase activity of the yeast control strain harboring plasmids pGADT7-T and pGBK-T7-53 (S-40 + p53), which encode the SV40 T antigen and the p53 protein (Figure 2), indicating an interaction between AppA and CryB. Interaction studies in the identical yeast system revealed 35% of the control for the two component system proteins RegA and RegB of *Rhodobacter capsulatus* and ~15% for RegA and NtrX, which were shown to interact *in vitro* in a pull-down assay (29). Cotransfer of pGBK-T7-*cryB* and pGAD-T7-*ppsR* into *S. cervisiae* Y187 led to no measurable β-galactosidase activity.

CryB binds to the SCHIC domain of the AppA protein

To further elucidate which part of the AppA protein is involved in the interaction to the CryB protein, different
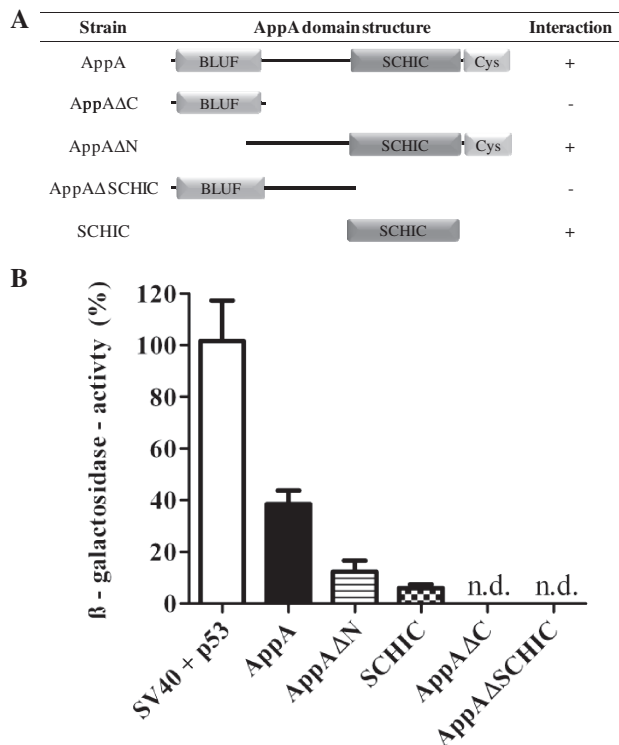
Figure 2. Interaction of CryB with different domains of AppA. (**A**) Schematic presentation of the AppA domains used in the yeast two-hybrid assay. +, growth of the transformants on selective agar indicates an interaction; −, no growth on selective agar. (**B**) Quantitative β-galactosidase activity assay for at least three yeast two-hybrid clones. The internal control SV40 T antigen together with p53 protein was set to 100%. The other β-galactosidase activities were compared to the control. n.d., no detectable β-galactosidase activity. Mean values of three different experiments and the standard deviations are shown.

domains of AppA (Figure 2A) were cloned into the pGAD-T7 vector and cotransformed into *S. cervisiae* strain AH109. Yeast cells harboring CryB and the C-terminal part of the AppA protein (AppAΔN) showed strong growth on the stringent selection media (leucine/ tryptophan/histidine and adenine), whereas cotransformation with the SCHIC domain of AppA alone (SCHIC) resulted in weaker growth. No growth on leucine/tryptophan/histidine was visible after cotransformation with the plasmids containing the BLUF domain alone (AppAΔC) or the N-terminal part of the protein, lacking the SCHIC domain (AppAΔSCHIC). After the transfer into *S. cervisiae* Y187 β-galactosidase activity of ~15% for AppAΔN and 8% for the SCHIC domain of AppA was measured (compared to the SV40/p53 control strain), while strains containing AppAΔC or AppAΔSCHIC showed no activity (Figure 2B).

To further verify the interaction indicated by the yeast two-hybrid assay pull-down analyzes were performed. To this end full-length AppA protein tagged with MBP (14) was bound to amylose resin and a pull-down with cell lysate from *R. sphaeroides* was performed. Since the levels of CryB protein in the wild-type are low, an *R. sphaeroides* His-CryB overexpression strain harboring the pRK*pufcry* plasmid was used for the pull-down (15). After incubation with the cell lysate and extensive washing under reducing conditions, high amounts of the CryB protein were eluted together with the full-length AppA protein (Figure 3A), while no CryB was visible on western blot in the elution fractions without prior binding of AppA to the amylose resin (Figure 3B). The results from the yeast two-hybrid assays suggested that the C-terminal part and the SCHIC domain of the AppA protein are sufficient for interaction with CryB. To confirm this interaction GST tagged AppAΔN (13) or the AppA SCHIC domain were bound to gluthathion sepharose and a pull-down with cell lysate from the CryB overexpression was performed. Again CryB protein was detectable on western blot in the elution fractions (Figures 3C and D). Control experiments without prior binding of the AppA domains showed no signals on western blot. Reconstitution of the purified MBP-AppA, GST-AppAΔN and GST-SCHIC with heme as described before (13) had no influence on the interaction. To eliminate the possibility of an unspecific binding of the His-tag of CryB to the GST-AppAΔN protein a control experiment was performed using cell lysate from the *R. sphaeroides* His-LOV overexpression strain harboring the pRK*puflov* plasmid (7). No LOV protein could be detected on the western blot (Figure 3E).
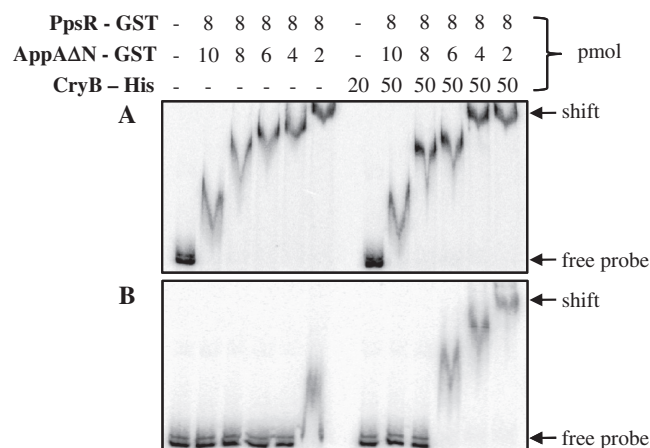
Both proteins CryB and AppA form aggregates in high concentration, which excludes many methods for determination of binding affinities. Therefore microscale thermophoresis (30) was used to quantitatively follow the interaction of the two proteins. This recently introduced method follows the directed motion of molecules by temperature gradients (thermophoresis), which is influenced by the presence of interaction partners. By labeling either CryB and adding AppA in different concentrations or vice versa a K_D of 595 ± 397 nM was determined (Supplementary Figure S1).
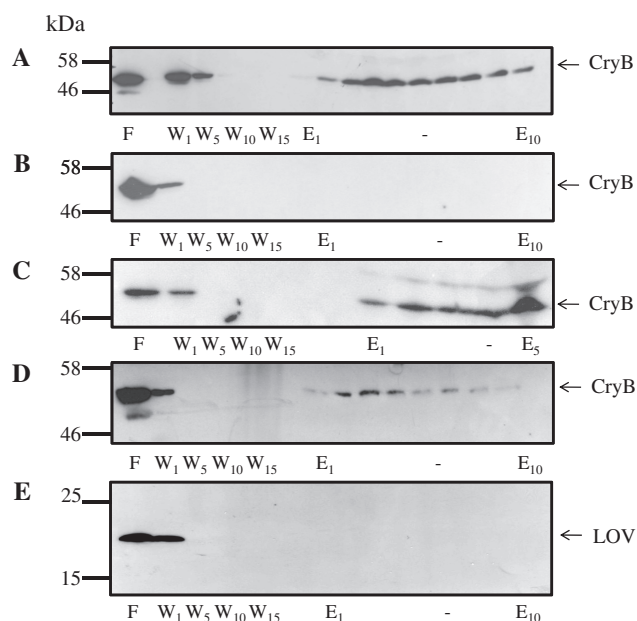
Figure 3. *In vitro* interaction of CryB and AppA. Western blots of 12% SDS–PAGE from [glutathione S transferase (GST)- and MBP-] pull-down assays using a CryB-specific antibody (A–D) or a LOV-specific antibody (E). (**A**) AppA-MBP protein bound to amylose–agarose and incubated with cell lysate from *R.s.* $\Delta cryB$(pRK*pufcryB*). (**B**) Incubation of cell lysate from *R.s.* $\Delta cryB$(pRK*pufcryB*) with amylose–agarose. (**C**) GST-AppAΔN bound to glutathione-sepharose and incubated with cell lysate from *R.s.* $\Delta cryB$(pRK*pufcryB*). (**D**) GST-SCHIC bound to glutathione–sepharose and incubated with cell lysate from *R.s.* $\Delta cryB$(pRK*pufcryB*). (**E**) AppA-MBP protein bound to amylose–agarose and incubated with cell lysate from *R.s.* 2.4.1(pRK*puflov*). F, cell lysate flow through; W, washing fractions (same volume as F); E, elution fractions (same volume as F).

Figure 4. CryB affects AppA–PsR interaction. Mobility shift assay showing the influence of AppAΔN on the DNA-binding activity of PsR (left) and the effect of CryB on this interaction (right). The samples were analyzed on a 4% native polyacrylamide gel. (**A**) Incubation and gel run were performed in the dark or under red light. One millimolar H_2O_2 was added to the binding buffer. (**B**) Incubation and gel run were performed in the dark or under red light. One hundred millimolars of DTT in binding buffer.

bound to the DNA probe (data not shown). Since the effect was rather small and statistical quantification was not possible, we used the yeast two-hybrid system to further investigate the effect of light in CryB–AppA interaction (see below). Addition of increasing amounts of His-CryB to equal amounts of GST-PsR and GST-AppAΔN under reducing conditions again led to a release of AppA/PsR interaction visible through an increase of probe retardation in the gel (Supplementary Figure S2).

Light-dependent interaction of CryB and AppA in yeast

The *in vitro* methods do not allow to mimic the light and redox situation, which is present within the cells. In order to further analyze the light-dependent interaction of CryB and AppA we investigated the effect of blue light on the interaction of the two proteins in yeast. The yeast strain harboring pGBK-T7-*cryB* and pGAD-T7-*appAΔN* was grown over night and the culture was diluted to an OD_{600} of $\sim$0.15 and grown for another 2 h in the dark at $\sim$90 μmol diluted oxygen. Then the culture was split and half of the culture was illuminated with blue light (20 μmol m^{-2} s^{-1}) the other half was kept in the dark. Samples were taken at various time points and used to quantify the interaction by measuring the β-galactosidase activity. As seen in Figure 5 the β-galactosidase activity increased over time by an average factor of almost two, when the culture was illuminated, while only a slight increase (factor less than 1.2) was observed in the dark. As a control the same experiment was performed with the yeast control strain harboring plasmids pGADT7-T and pGBK-T7-53, which encode the SV40 T antigen and the p53 protein. No light effect on the interaction of these control proteins was observed (Figure 5).

CryB inhibits AppA binding to PsR

In earlier studies we showed that the C-terminus of AppA interferes with binding of PsR to the *puc* promoter (13). The *puc* genes encode proteins of the light harvesting II complex. In order to test a possible influence of the CryB protein on the interaction of these two proteins, equal concentrations of GST-PsR were incubated with the radioactively labeled DNA probe, while adding increasing amounts of GST-AppAΔN with or without an excess of His-CryB. 8 pmol of GST-PsR were sufficient for full retardation of the DNA in the gel (Figure 4) under oxidizing conditions. Addition of GST-AppAΔN released GST-PsR from the DNA resulting in an increase of free probe (Figure 4A). No effect of CryB on this release was observed under oxidizing conditions no matter whether experiments were performed in the dark (Figure 4A) or under white light (not shown). Under reducing conditions (Figure 4B) only a molar excess of GST-PsR to GST-AppAΔN (4:1) led to a weak retardation of the probe. Addition of an excess of CryB (50 pmol) under reducing conditions favored the release of PsR from the interaction with AppAΔN, which is visible in an increased shift of the probe (Figure 4B). Illumination of the samples and the gel with white light slightly decreased the amount of free GST-PsR that was
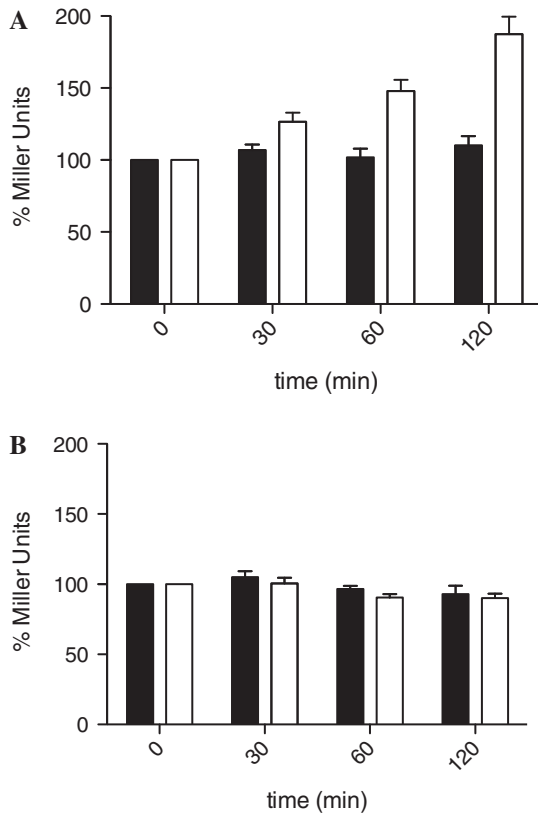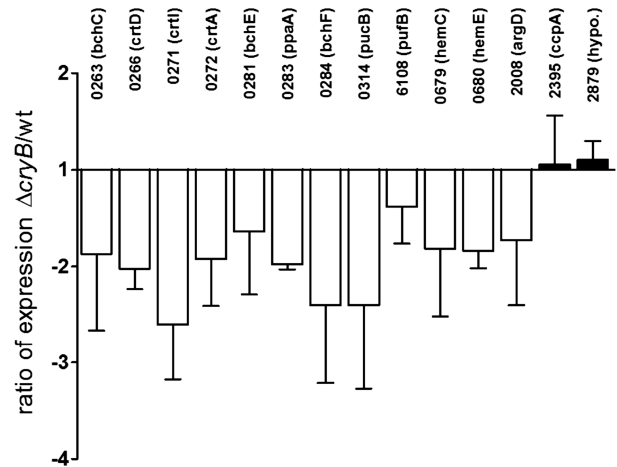
Figure 5. Light-dependent interaction of CryB and AppAΔN in the yeast two-hybrid system. Yeast strains were cultivated in the dark at ~90 µmol dissolved oxygen to an OD_{600} of 0.2–0.25. Then half of the culture was illuminated with blue light (white bars) and half of the culture was kept in the dark (black bars). (**A**) Yeast strain harboring pGBK-T7-*cryB* and pGAD-T7-*appAΔN*. Percentage Miller Units represent the mean of five to six independent experiments and the standard deviation is indicated. (**B**) yeast control strain harboring plasmids pGADT7-T and pGBK-T7-53. Percentage Miller Units represent the mean of five independent experiments and the standard deviation is indicated. Miller Units at time point zero equal 100%.

Figure 6. Expression changes of selected genes as determined by real-time RT–PCR. Bars indicate gene expression in the *cryB* deletion mutant compared to the wild-type after 60 min blue light treatment under semiaerobic growth conditions. White bars correspond to genes of the PpsR operon, bars in black depict control genes of the PrrA operon (31,32) Numbers correspond to *R. sphaeroides* gene annotations. RSP_2879, putative uncharacterized protein. The mean of three experiments is given and standard deviation is indicated.

CryB affects expression of all genes of the PpsR regulon

Our earlier observation that CryB affects the expression of *puf* and *puc* genes (15) is in agreement with our finding that CryB modulates binding of PpsR to target sequences, since *puf* and *puc* genes are part of the PpsR regulon. Consequently, CryB should also affect the expression of all other members of the PpsR regulon (31,32). In order to verify this, the expression levels of these genes were compared in the wild-type 2.4.1 and in the mutant 2.4.1ΔcryB (15). The differences in expression levels between the two strains when grown under microaerobic conditions were too low to reveal reproducible and significant results. We therefore quantified gene expression in cultures illuminated with blue light for 60 min by real-time RT–PCR. As shown in Figure 6 all genes belonging to the PpsR regulon were affected by the lack of CryB. The expression levels in the mutant strain were 1.4–2.6-fold lower than in the isogenic wild-type strain. Most of these genes encode genes required for the synthesis of pigment binding proteins (*puf, puc*), synthesis of bacteriochlorophyll (*bch*) or carotenoids (*crt*) and are clustered on the chromosome. Genes *hemC* and *hemE* required for heme synthesis and *argD* (acetyl ornithine aminotransferase) are localized in distinct regions on the chromosome but show similar CryB dependence. We included two genes in our study, which are part of the PrrA regulon (32) but not of the PpsR regulon, *ccpA* and RSP2877. PrrA is another important regulator of photosynthesis genes, including the *puf* and *puc* operons and induces transcription at low oxygen tension. The expression levels of these two genes did not differ significantly in the two strains excluding the possibility that CryB affects photosynthesis gene expression solely through PrrA or has a general, unspecific effect on gene expression.

DISCUSSION

In previous studies we identified and characterized the unusual cryptochrome CryB in *R. sphaeroides* (15). While photoreactivation was slightly influenced in a CryB mutant strain (33) and a high binding affinity of CryB towards single-stranded DNA (with T<>T dimers) was observed, the protein showed no repair activity *in vitro* and an overall low homology to other cryptochromes/photolyases (15). An effect on the expression of the photosynthesis genes was clearly visible in a CryB mutant strain that comprised a slightly lower pigmentation than the wild-type, while an overexpression of the protein led to even stronger reduction in pigmentation (15). Since the affinity of CryB to double-stranded DNA was low, we considered that it may act on gene expression by interaction to other proteins (15). To further elucidate the signal chain leading from CryB to photosynthesis gene expression, a yeast two-hybrid approach was used to test interaction against known

regulators of the photosynthesis genes. The role of the AppA/PpsR system in light and redox regulated photosynthesis gene expression is well established [(4,13,34,35); Figure 1]. While tests for an interaction between PpsR and CryB were negative, an interaction with AppA could be verified in the yeast two-hybrid analysis (Figure 2) and by pull-down assays (Figure 3A). Physical interaction between photoreceptors, namely cryptochromes and phytochromes has been reported in plants for over a decade (36–38), with coprecipitation proven for *Arabidopsis thaliana* cry2 and phyB (36). The latter study demonstrated a role of genetic interaction between the *Arabidopsis* photoreceptors phyB and cry2 in the control of flowering time, hypocotyl elongation and circadian period. Microscale thermophoresis also confirmed interaction of CryB and AppA *in vitro* and revealed a K_D of ~0.6 μM. This is in a similar or lower range than observed for the interaction of other photoreceptor–protein interactions. For the sensory rhodopsin and transducer II of *Halobacterium salinarum* a K_D in the micromolar range was shown (39). A K_D of 8 μM was determined for the interaction of the *Anabaena* sensory rhodopsin and the tetramer of its cognate transducer (40). The C-terminal tails of mammalian cryptochromes Cry1 and Cry2 interact with the transcription factor mBMAL1 with K_D values of ~10 μM (41). However, one has to keep in mind that all these *in vitro* affinities may not fully reflect the *in vivo* situation. On one hand other cellular components may influence the interaction between the proteins *in vivo*, on the other hand temporary localization of the binding partners, as demonstrated for many proteins in bacteria over the last years, can cause high local concentrations.

Subsequently, we tested the interaction of different AppA domains with the CryB protein. The C-terminal part of AppA (AppAΔN) comprising the heme-binding SCHIC domain proved to be sufficient for the interaction (Figures 2 and 3C). However, the SCHIC domain alone (GST-SCHIC) showed a weaker binding to CryB than AppAΔN or the full-length AppA protein (Figure 2) indicating that protein parts flanking the SCHIC domain strengthen its binding to CryB. The AppA SCHIC domain is a member of a newly discovered group of heme-binding oxygen sensors that react to an oxidation by discoordination of the heme cofactor (14). When isolated from *E. coli*, only a very low percentage of AppA carried the heme cofactor, but incubation with hemin considerably increased this percentage to ~30% (13). In this study, the same amount of CryB was bound to AppA no matter whether it was reconstituted with hemin or not. This excludes that binding of CryB to AppAΔN is solely mediated by the heme cofactor.

In order to affect expression of photosynthesis genes through AppA, CryB would need to influence AppA–PpsR interaction. To test this possibility electrophoretic mobility shift assays with the upstream region of the *pucB* gene together with PpsR and different amounts of AppA and CryB were performed (Figure 3). Addition of CryB under oxidizing conditions had no effect on the interaction between AppAΔN and PpsR (Figure 4A). Interestingly, a molar excess of CryB led to an increase

of free PpsR protein under reducing conditions in the dark (Figure 4B and Supplementary Figure S2). This is in accordance with our observation that the *R. sphaeroides* overexpression strain Δ*cryB* (pRK*pufcryB*) shows diminished levels of photosynthesis gene expression and lowered absorption spectra (15).

We could demonstrate a light-dependent interaction of CryB and AppA *in vivo* in the yeast system. Since we used the yeast strain expressing the C-terminal part of AppA we can exclude that this light effect was due to the BLUF domain of AppA. The *in vivo* experiments revealed an increase of the interaction after illumination. This would lead to decreased binding of PpsR by AppA and consequently decreased expression of photosynthesis genes in response to blue light. Thus CryB would support the light-dependent effect of AppA on photosynthesis gene expression.

Our previous *in vivo* study revealed similar effects on photosynthesis gene expression of an overexpression of CryB and of a lack of CryB (15). This implies that further cellular factors influence the CryB-dependent signaling *in vivo* and maybe also its interaction to AppA or the interaction of AppA to PpsR, when CryB is bound. CryB does not only affect photosynthesis genes but also expression of many other genes as revealed by a recent transcriptome study (42). CryB influences blue light as well as oxidative stress dependent gene expression but also gene expression in the dark. The recently solved structure of CryB (43) identified an iron-sulfur cluster as third cofactor. It is likely that this iron-sulfur cluster mediates redox-dependent effects of CryB. Among the genes affected by CryB are several genes for transcriptional regulators including *prrA* and also the *hfq* gene [(42), Supplementary Figure S3]. The data in Figure 6 demonstrate that CryB does not affect all genes of the PrrA regulon but we cannot exclude that altered expression of the *prrA* gene also has some influence on photosynthesis gene expression. The RNA chaperone Hfq was recently shown to also affect formation of photosynthetic complexes in *R. sphaeroides* (44). It is conceivable that CryB stimulates photosynthesis gene expression through certain signaling pathways but counteracts this stimulation through other pathways in order to contribute to balanced expression of these genes (Supplementary Figure S3). Thus deletion of the *cryB* gene or overexpression could result in similar phenotypes.

In summary, our data clearly demonstrate that CryB can bind to the AppA protein in a light-dependent manner and influence its affinity towards the transcriptional regulator PpsR. The rather limited *in vitro* and *in vivo* effects imply that CryB acts as a modulator of the AppA/PpsR system. It becomes clear that light-dependent regulation of bacterial photosynthesis genes involves a complex regulatory network including multiple photoreceptors in order to adapt appropriately to changes in environment.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–2 and Supplementary Figures 1–3.

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