Fungal cryptochrome with DNA repair activity reveals an early stage in cryptochrome evolution

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DASH (*Drosophila*, *Arabidopsis*, *Synechocystis*, Human)-type cryptochromes (cry-DASH) belong to a family of flavoproteins acting as repair enzymes for UV-B–induced DNA lesions (photolyases) or as UV-A/blue light photoreceptors (cryptochromes). They are present in plants, bacteria, various vertebrates, and fungi and were originally considered as sensory photoreceptors because of their incapability to repair cyclobutane pyrimidine dimer (CPD) lesions in duplex DNA. However, cry-DASH can repair CPDs in single-stranded DNA, but their role in DNA repair in vivo remains to be clarified. The genome of the fungus *Phycomyces blakesleeanus* contains a single gene for a protein of the cryptochrome/photolyase family (CPF) encoding a cry-DASH, *cryA*, despite its ability to photoreactivate. Here, we show that *cryA* expression is induced by blue light in a Mad complex-dependent manner. Moreover, we demonstrate that CryA is capable of binding flavin (FAD) and methenyltetrahydrofolate (MTHF), fully complements the *Escherichia coli* photolyase mutant and repairs in vitro CPD lesions in single-stranded and double-stranded DNA with the same efficiency. These results support a role for *Phycomyces* cry-DASH as a photolyase and suggest a similar role for cry-DASH in mucoromycotina fungi.

Phycomyces | cryptochrome | photolyase | evolution | mucoromycotina

Cryptochromes are flavoprotein photoreceptors identified in species belonging to all three domains of life (1–4). Cryptochromes sense blue light to regulate plant development and the circadian clock (5, 6). Moreover, they are an essential part of the mammalian circadian clock (3) and are proposed to act as magnetoreceptors in migratory birds (7). Cryptochromes are structurally related to photolyases (8), ancient flavoproteins that catalyze light-dependent DNA repair (9, 10), formerly described as photoreactivation of cells (11). The CPF is a diversified family of photoactive molecules with three major groups: the CPD photolyases, the (6, 4)-photolyases and the cryptochromes (3). Besides sharing similar photoactive domains, cryptochromes and photolyases bind the same cofactors, leading to the suggestion that photolyases are ancestors of cryptochromes (3, 10, 12). The common cofactor of cryptochromes and photolyases is noncovalently bound flavin adenine dinucleotide (FAD). In addition, secondary cofactors such as methenyltetrahydrofolate (MTHF), deazariboflavin, and others were identified bound to phytolyases and cryptochromes and should act as antenna chromophores (10, 13–15). Cryptochromes are distinguished from photolyases by carboxyl-terminal extensions of variable length. The accepted definition of a cryptochrome is therefore a protein with similarity to photolyases that has lost or reduced DNA repair activity and has gained a novel role in signaling (3). However, there are several examples of photolyases, including some from fungi, having a dual function as DNA-repair enzyme and photoreceptor (16–18).

Phylogenetic analysis of cryptochromes suggests that they can be grouped in three classes: plant cryptochromes, animal cryptochromes, and DASH (*Drosophila*, *Arabidopsis*, *Synechocystis*, Human)-type cryptochromes (cry-DASH). Cryptochromes from plants and many animals act as photoreceptors and lack DNA repair activity (3). However, cry-DASH have structural and photochemical properties more similar to photolyases, retain constricted DNA repair activity, and their possible roles in signaling are not yet well established. Cry-DASH were shown to bind and repair CPDs in single-stranded DNA or loop-structured duplex DNA (19, 20), but repair of CPDs in duplex DNA by cry-DASH has only been suggested from in vitro studies (e.g., ref. 21). Cry-DASH may therefore represent an evolutionary intermediate between cryptochromes and photolyases (3, 22).

Most fungi perceive blue light by using homologs of the White Collar (WC) Complex, a photoreceptor and transcription factor complex that was first identified in the fungus *Neurospora crassa* (23). In addition, *N. crassa* has two members of the CPF: a photolyase, PHR, that is required for light-dependent DNA repair (24), and a cry-DASH, CRY, that is capable of binding single-stranded and double-stranded DNA or RNA, but does not provide photorepair activity in vivo (25). A role for CRY in sensory perception has been proposed based on the alteration of light-dependent transcription for some genes in the *cry* mutant (26). In addition, CRY participates in the regulation of the circadian clock through a CRY-dependent oscillator (27). Cry-DASH have been described in other fungi and they have minor sensory roles. They participate in the regulation by light of development in *Sclerotinia sclerotiorum* and *Fusarium fujikuroi* (28, 29), and pigment accumulation in *F. fujikuroi* (29).

Significance

Photolyases repair UV-B–induced DNA lesions. They form a large protein family together with cryptochrome photoreceptors (cryptochrome/photolyase family, CPF). A more recently discovered CPF subclade consists of DASH (*Drosophila*, *Arabidopsis*, *Synechocystis*, Human)-type cryptochromes (cry-DASH), present in bacteria, plants, animals, and fungi. Cry-DASH are considered as photoreceptors with residual repair activity for DNA lesions in single-stranded DNA. Canonical photolyases repair such lesions in single-stranded and double-stranded DNA. Here, we show that mucoromycotina fungi except *Umbelopsis ramanniana*, which is an early diverging lineage within the mucoralean fungi, encode only cry-DASH. They possess the full spectrum of DNA repair activity as canonical photolyases as exemplified for the *Phycomyces* CryA. This finding is a unique example of CPF evolution where a canonical CPD-photolyase was lost but its function was maintained by cry-DASH.

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The fungus *Phycomyces blakesleeanus* belongs to the mucoromycotina, a basal fungal clade and has been used as a model organism to investigate the responses of fungi to light (30). *Phycomyces* senses blue light through the action of the Mad complex, a homolog of the WC complex in *N. crassa* (31, 32). Other elements of the signal transduction pathway for the phototropism of the fruiting body have been identified by mutations, but the corresponding *mad* genes remain to be identified (33). *Phycomyces* shows photoreactivation under near-UV/blue light, and this response requires the activity of the Mad complex (34), strongly suggesting that *Phycomyces* has a functional DNA photolyase.

We have found a gene for a cry-DASH, *cryA*, in the genome of *P. blakesleeanus*. Unlike Ascomycetes and Basidiomycetes, the genome of *Phycomyces* does not contain genes for other cryptochromes or photolyases despite the observation of photoreactivation. Here, we describe the function of CryA from *Phycomyces* as a DNA-repair enzyme and its regulation by light and its expression during vegetative growth. We propose that *Phycomyces* CryA represents an ancient stage in the evolution of cry-DASH.

Results

A cry-DASH Gene in *Phycomyces*. The observation of photoreactivation in *Phycomyces* suggested the action of a photolyase in this species (34). Searching the *Phycomyces* genome database for genes encoding proteins of the CPF, we found only one gene encoding a protein (ID no. 85761) similar to *N. crassa* CRY (25) and *S. sclerotiorum* Cry1 (28). The absence of a canonical photolyase gene in the *Phycomyces* genome suggested that the cryptochrome gene, *cryA*, could encode a protein that acts as a photolyase. The *Phycomyces* cryptochrome gene has a length of 2,114 bp and contains three introns of 220, 68, and 62 bp, respectively. CryA has a predicted length of 587 amino acids and is similar to other fungal cryptochromes. For example, CryA is 48.6% identical to *Neurospora* CRY and 47.7% to *S. sclerotiorum* Cry1. To rule out a sensory role for CryA, we sequenced *cryA* in strains with mutations in *mad* genes that modify phototropism of the fruiting body (33). We did not detect any difference in *cryA* in the *mad* mutants other than several polymorphisms observed in wild-type strains and mutants derived from them (*SI Appendix*, Table S1).

Genes encoding CPF members are found in different taxonomic groups. To assign *Phycomyces* CryA to one of the CPF subgroups we built a phylogenetic tree with sequences from several organisms (Fig. 1). *Phycomyces* CryA clustered with other members of the cry-DASH subgroup, supporting the proposal that CryA is a cry-DASH. We identified cry-DASH genes in the genomes of other mucoromycotina fungi, including *Backusella circina*, *Mucor circinelloides*, and *Rhizopus delemar*. We found a class I CPD photolyase gene in the genome of *Umbelopsis ramaniana*, but we did not identify any gene for a member of the CPF in the genomes of *Rhizopus microsporus* or *Lichtheimia hyalospora* (*SI Appendix*, Fig. S1). Our analysis suggests that the common ancestor of the mucoromycotina fungi had genes for a CPD-photolyase and a cry-DASH and that these genes have been retained or lost during their evolution.

We aligned the amino acid sequence of *Phycomyces* CryA with that of *Arabidopsis thaliana Cry3*, one of the best-characterized members in this family, together with cry-DASH from *Xenopus*, zebrafish, and *Neurospora*, to detect the conservation of functional regions in these proteins. All of the amino acids that act in binding of FAD in *Arabidopsis* Cry3 (20, 35) or are essential for hydrogen bonding of MTHF are conserved across the five species, suggesting that *Phycomyces* CryA might bind FAD and MTHF in a manner similar to that of other members in the cry-DASH subfamily (35, 36) (*SI Appendix*, Fig. S2). The cocrystal structure of *Arabidopsis* Cry3 in complex with a T5 oligonucleotide containing a repaired T<>T lesion revealed similarities but also functional differences between lesion-binding active sites of conventional class I CPD photolyases and cry-DASH (20). All of the functionally important residues shared between class I CPD photolyases and cry-DASH are conserved in *Phycomyces* CryA (*SI Appendix*, Fig. S3). A modeled structure of *Phycomyces* CryA using the structure of *Arabidopsis* Cry3 in complex with the T5 oligonucleotide as template supports the assumption that *Phycomyces* CryA is able to bind CPD-lesions (*SI Appendix*, Fig. S3).

The Gene *cryA* is Induced by Blue Light Through the Activity of the Mad Complex. The enzyme responsible for photoreactivation in *Phycomyces* is induced by light, and light-induction requires the Mad complex (34). If CryA is responsible for photoreactivation, then the gene should be induced by light in a Mad complex-dependent manner. We assayed the effect of light exposure on *cryA* expression and found that blue light increased the accumulation of *cryA* mRNA 15-fold, but red light had no effect (Fig. 2*A*). Maximum mRNA levels of *cryA* were obtained after 30 min of illumination. Longer exposures reduced the mRNA levels because of photoadaptation, but higher levels than in darkness were still detectable after 4 h of light (*SI Appendix*, Fig. S4*A*). The threshold for the photoactivation of *cryA* expression was

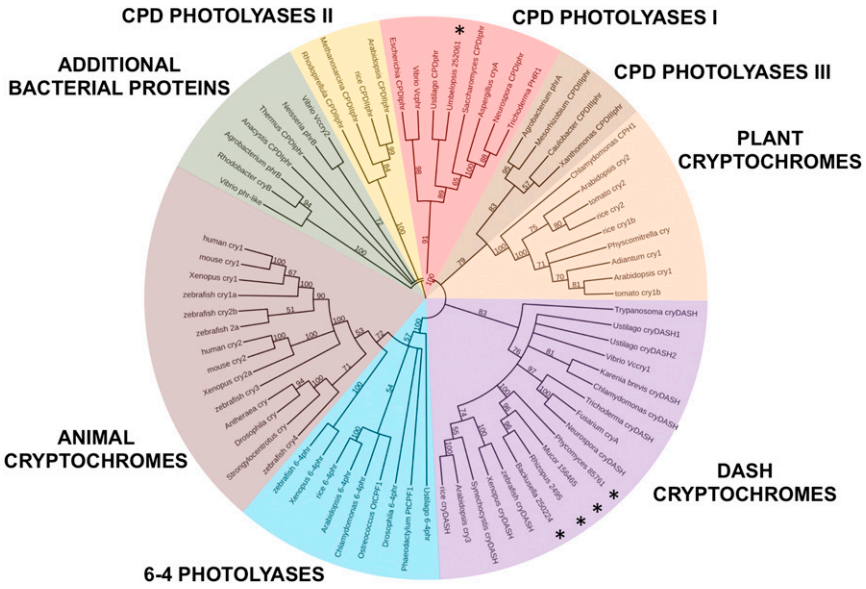

Fig. 1. Phylogenetic analysis of the cryptochrome/photolyase family. Cladogram representing the distribution and evolutionary relationships of members of the photolyase/cryptochrome family in a broad range of organisms. The eight subfamilies are indicated with different colors, following the classification of Chaves et al. (3). Asterisks mark the position of members of the photolyase/cryptochrome family in mucoromycotina fungi. The phylogenetic tree is midpoint rooted and has been obtained by using the neighbor-joining method with nodal support referred to 1,000 pseudoreplicates. Only the topology is presented, and branches are not proportional to the amount of evolutionary change that has taken place along them. Bootstrap values more than 50% are shown at the respective nodes. Bootstrap nodes under 50% have been collapsed.

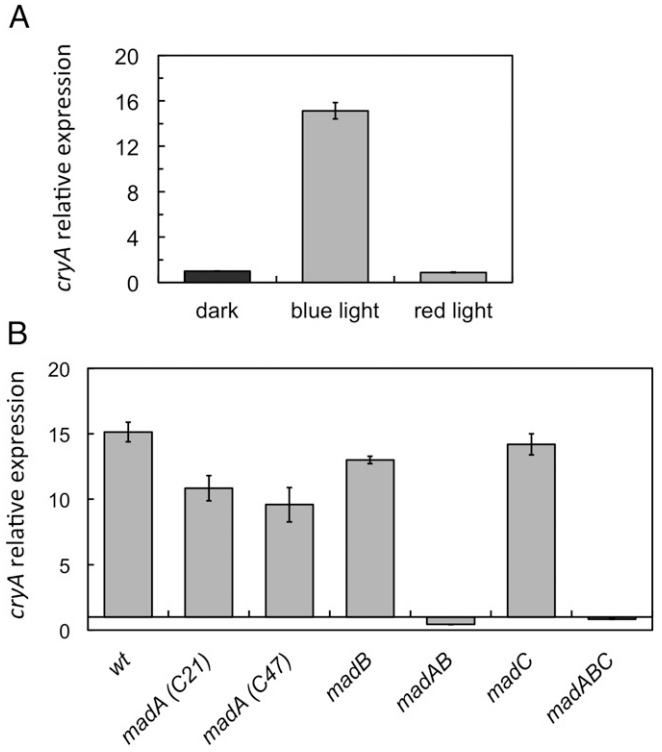

Fig. 2. Regulation of *cryA* by light. Total RNAs were isolated from mycelia exposed to light or kept in the dark. The amount of mRNA for each gene was assayed by quantitative RT-PCR. Each fluorescent signal was first normalized to the corresponding actin (*actA*) signal and then was normalized to the signal obtained in the dark. Given are means and SEMs from 3 to 12 independent experiments. (*A*) Expression of *cryA* in mycelia exposed to blue (4.7×10^3 J·m^{-2}) or red light (3.2×10^3 J·m^{-2}) or kept in the dark. (*B*) Photoactivation of *cryA* expression in the wild type and *mad* mutants after 30 min of blue light irradiation (4.7×10^3 J·m^{-2}). The *madA madB* double mutant is labeled as *madAB*, and the *madA madB madC* triple mutant is labeled as *madABC*.

1 J·m^{-2} (*SI Appendix*, Fig. S4*B*), similar to the threshold for the photoactivation of the photoreceptor gene *wcoB* but 10^4 times higher than the threshold for the photoactivation of other *Phycomyces* photoreceptor genes (32). The high threshold ensures that CryA only accumulates when the fungus is exposed to high intensities of light and is consistent with a role as photolyase to repair UV-damaged DNA.

The perception of light in *Phycomyces* requires the products of the *madA* and *madB* genes that interact to form a photoreceptor complex (31, 32). Strains with mutations in *madA* or *madB* had a mild defect in light-dependent *cryA* mRNA accumulation. Similar mild effects on photoactivation of transcription have been observed for other *Phycomyces* genes and are probably due to the high intensity of light that is applied and to the fact that none of them is a complete knockout (31, 32). However, the double mutant strains did not show any accumulation of *cryA* mRNA after light exposure (Fig. 2*B*). A mutation in *madC* did not affect the regulation of *cryA* by light because this gene participates in the regulation of phototropism (33). The accumulation of CryA protein followed the behavior of *cryA* mRNA. We detected CryA in mycelia kept in the dark, and the amount increased after exposure to light with a maximum after 2 h (*SI Appendix*, Fig. S4*C*). The light-dependent accumulation of CryA was not observed in the *madA madB* double mutant (*SI Appendix*, Fig. S4*C*). We conclude that the Mad complex is required for the activation of *cryA* by light.

CryA Complements Photolyase-Deficient *E. coli* Cells. CryA is the only member of the photolyase/cryptochrome family in *Phycomyces*, and *Phycomyces* shows photoreactivation (34). Therefore, we reasoned that the cry-DASH encoded by *cryA* might act as a functional photolyase. As an initial test for photolyase function, we expressed CryA in the photolyase-deficient KY1225 *E. coli* strain (37) to see whether CryA can complement the UV-B hypersensitivity of this mutant. This approach was used in several other studies to study photolyase function of CPF members (38, 39). As controls we included KY1225 cells carrying the empty expression vector or cells expressing *E. coli* photolyase (PHR) from the same vector as CryA (Fig. 3). The UV-B–treated KY1225 photolyase mutant carrying the empty vector did not show a significant increase in the number of survivors after irradiation with photoreactivating white light unlike the corresponding photolyase-proficient KY1056 strain, as expected. Using the same light treatments, cells expressing CryA showed a similar percentage of survivors as those cells expressing *E. coli* photolyase. Our results indicate that CryA has photolyase activity that complements the absence of the *E. coli* photolyase PHR. However, a direct comparison of the repair efficiencies of CryA and PHR by this assay is difficult, because the expression levels of the two proteins were not the same (*SI Appendix*, Fig. S6) although expression was driven by the same promoter. The only difference between CryA and PHR-expressing KY1225 cells was a higher survival rate of the latter when no photoreactivating light was given after the UV-B exposure (Fig. 3).

CryA Binds Flavin and Methenyltetrahydrofolate. A requirement for photolyase and cryptochrome photoreceptor function is binding of the FAD cofactor (10). Our attempt to express recombinant CryA protein as a 6xHis-tag fusion in *E. coli* BL21 cells and to purify cofactor-containing protein to homogeneity failed for unknown reasons. Thus, we characterized CryA from extracts of photolyase-deficient KY1225 *E. coli* cells that provided soluble (*SI Appendix*, Fig. S6) and functional (Fig. 3) protein. As controls we

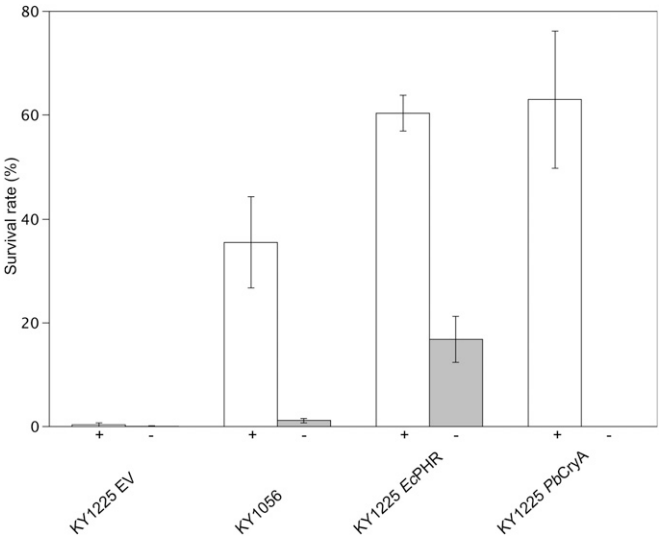

Fig. 3. Survival rates of *E. coli* wild type and photolyase mutant strains. Dilutions of cells of the different genotypes were spread on LB plates and treated with UV (for spectrum, see *SI Appendix*, Fig. S5) for 5 s. Afterward, plates received 1 h of photoreactivating white light (open bars, +) or were directly transferred to darkness (gray bars, –). Formed colonies were counted after incubation of the plates at 37 °C in darkness for 16 h. Ratios of colony numbers from UV-treated cells to cells not treated with UV are given as survival rates (in %). KY1225 EV, photolyase mutant transformed with empty pQE-30 vector; KY1056, wild type; KY1225 *Ec*PHR, photolyase mutant expressing *E. coli* photolyase; KY1225 *Pb*CryA, photolyase mutant expressing *P. blakesleeanus* CryA. Given are means and SEs of at least three biological replicates.

included KY1225 cells expressing *E. coli* PHR and cells carrying the empty expression vectors. Total protein extracts from these cells were analyzed spectroscopically (Fig. 4). Extracts from CryA-expressing cells showed a higher absorbance than the control between 300 nm and 500 nm (Fig. 4 *A* and *B*), indicative for the presence of folate and flavin cofactors. Compared with CryA, the expression level of PHR was lower (*SI Appendix*, Fig. S6). To obtain more convincing information about the cofactors bound to CryA, we applied fluorescence spectroscopy. Extracts of CryA-expressing cells showed pronounced excitation and emission spectra (Fig. 4*C*) not detectable for the negative control (Fig. 4*D*). Thus, the difference spectra of extracts from CryA-expressing cells and the negative control shown in Fig. 4*E* are essentially the same as those shown in Fig. 4*C*. The emission peak at 520 nm with excitation at 450 nm (Fig. 4*C*, dashed line) demonstrates the presence of FAD_{ox} (40). Excitation at 380 nm, which is the peak absorbance of MTHF in photolyase and cry-DASH (41, 42) results in strong emission with a peak at 476 nm (Fig. 4*C*, solid line). The peak position at 476 nm is most likely caused by a mixture of emission from FAD_{ox} (peak at 520 nm) and MTHF (broad peak at 460 nm) (43). The presence of MTHF in CryA is further confirmed by the excitation peak at 380 nm for 460 nm emission (Fig. 4*C*, dotted line). Moreover, the same 380 nm peak position for 520 nm emission would be in line with energy transfer between MTHF and FAD (Fig. 4*C*, red dotted line). From these results, we conclude that *Phycomyces* CryA binds FAD and MTHF, and that MTHF most likely acts as an antenna chromophore, which transfers excitation energy to FAD.

CryA Repairs CPD Lesions in ssDNA and dsDNA with Similar Efficiency. The data presented in Fig. 3 indicate a photolyase function for *Phycomyces* CryA. To analyze this activity further, we performed in vitro repair assays by using total protein extracts of KY1225 photolyase-deficient *E. coli* cells expressing CryA or PHR. As substrates we used a 50-mer oligonucleotide probe with a single T<>T lesion present in the VspI recognition sequence of single-stranded DNA (ssDNA), in a double-stranded DNA (dsDNA) or in a loop of duplex DNA. Cleavage of the recognition site by VspI is only possible after repair of the T<>T. Assays with CryA showed light-dependent repair of the CPD-lesion in ssDNA (Fig. 5*A*, blue solid line) and loop-structured duplex DNA (Fig. 5*A*, green solid line). In addition, we observed light-dependent repair of the CPD by CryA in dsDNA (Fig. 5*A*, red solid line), which was as efficient as in ssDNA. The repair of CPDs in dsDNA is consistent with the efficient complementation of the photolyase-deficient KY1225 *E. coli* strain by CryA (Fig. 3). As a control, we performed the same experiments with PHR (Fig. 5*B*) and obtained similar result as for CryA. In addition, to test that the duplex probe was not partially molten in two single strands, we characterized the repair of CPDs by the *Arabidopsis* cry-DASH Cry3. *Arabidopsis* Cry3 is unable to bind and repair CPDs in dsDNA (19, 20). As expected, *Arabidopsis* Cry3 repaired the T<>T in the ssDNA but not in dsDNA (*SI Appendix*, Fig. S9). Our results show that *Phycomyces* CryA is a cry-DASH with the capacity to repair CPDs to a similar extent in single- and double-stranded DNA and, thus, behaves like a conventional DNA photolyase.

Discussion

We have characterized a cry-DASH with a previously unidentified DNA photorepair activity in the fungus *P. blakesleeanus*. We propose that this enzyme plays a key role in the photoreactivation activity that has been described in *Phycomyces* spores (34). Because it is not possible to delete specific genes in *Phycomyces*, we could not create a Δ*cryA* mutant to test whether it lacks photoreactivation. However, our results open the way to further characterization of dsDNA repair activity in other members of the cry-DASH subfamily to clarify their relevance in DNA repair.

Our observation that a canonical photolyase gene was missing in the *Phycomyces* genome despite the described photoreactivation ability prompted our characterization of the DNA repair activity of CryA. Analysis of the transcriptional response of *cryA* demonstrated that the gene was induced by blue light and required the activity of the Mad complex. The same regulation has been proposed for the photoreactivation enzyme (34). Induction of photolyase expression by visible light has been described for other fungi, animals, and plants (17, 38, 44) and may serve to increase the amount of this repair system to cope with strong UV exposure. The single mutants of *madA* and *madB* still showed *cryA* induction in contrast to the double mutant. Because none of both *madA* and *madB* are full knockout alleles, we propose that both alleles provide residual activity, which is insufficient to induce *cryA* in the double mutant. Further confirmation of the photorepair activity of CryA was obtained by the complementation of the *E. coli phr* mutant by *Phycomyces cryA*. Cells expressing CryA showed photoreactivation with approximately 60% survival rate similar to *E. coli* cells expressing *E. coli* photolyase, PHR. The genotype of the *E. coli* KY1225 strain used for the complementation assays includes a null mutation of *phr* and a missense mutation in *recA* (*recA56*), a protein required for e.g.,

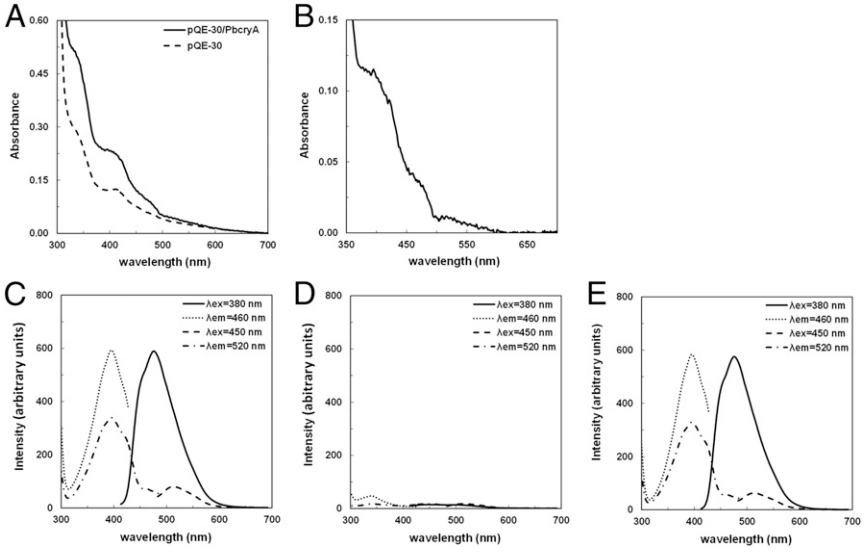

Fig. 4. Absorption and fluorescence spectroscopy of *E. coli* KY1225 protein extracts. (*A*) Absorption spectra measured in prepared extracts and normalized to 1 mg·ml^{-1} of total proteins. Solid line, extract from cells expressing *Phycomyces* CryA; dashed line, extract from cells transformed by empty pQE-30 vector. (*B*) Difference absorption spectrum calculated from spectra shown in *A*. (*C*) Fluorescence spectra of the extract from cells expressing CryA. Solid line, emission due to excitation at 380 nm; dotted line, excitation wavelengths responsible for emission at 460 nm; dashed line, emission due to excitation at 450 nm; dash-dotted line, excitation wavelengths responsible for emission at 520 nm. (*D*) Fluorescence spectra of the extract from cells transformed by the empty vector. Instrument sensitivity and line styles are the same as for *C*. (*E*) Difference spectra obtained by subtraction of spectra shown in *D* from the corresponding spectra shown in *C*.

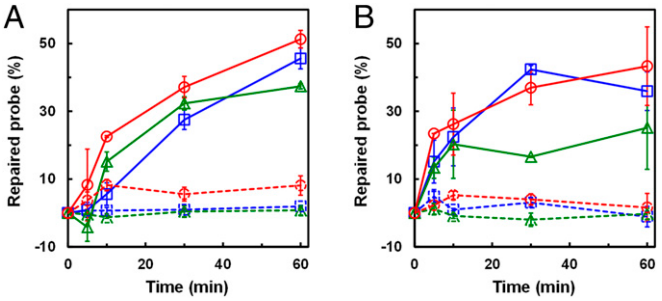

Fig. 5. Quantification of restriction-site restoration assays with protein extracts from *E. coli* KY1225-expressing *Phycomyces* CryA (*A*) or *E. coli* PHR (*B*). Percentage of repaired probe was calculated as described in *SI Appendix, SI Materials and Methods*. Blue squares, T<>T in the center of a single DNA strand; green triangles, T<>T within a mismatch loop in the center of DNA duplex; red circles, T<>T in the center of a well-formed DNA duplex. Full symbols and lines represent reactions irradiated with UV-A, dashed symbols and lines represent reactions kept in dark. Symbols stand for mean values and error bars indicate SEs of the two independent repetitions. Based on Western blots, the molar amount of CryA in *A* was approximately 100 times higher compared with that of PHR in *B*. The original data are shown in *SI Appendix*, Figs. S7 and S8.

homologous recombination repair. It is unlikely that the observed complementation was due to rescue of the *recA56* mutation by *cryA*. Similar experiments with cry-DASH from *N. crassa* failed to show photorepair activity in vivo (25). To our knowledge, *Phycomyces* CryA is the first cry-DASH that fully complements a photolyase-deficient *E. coli* strain and provides photorepair activity in vivo.

FAD is the essential cofactor of photolyases and cryptochromes. In most CPF members, a second chromophore acts as antenna to transfer excitation energy to FAD (10). Extracts from KY1225 cells expressing CryA showed maximum emission at 520 nm upon excitation at 450 nm, fully consistent with the presence of protein-bound FAD_{ox}. Based on this assay, we could not distinguish between FAD and other flavins such as FMN. However, all residues required for FAD binding are conserved in CryA, and only FAD has been described as catalytic cofactor or chromophore in photolyases and cryptochromes, respectively (10). Accordingly, we conclude that the emission of CryA at 520 nm upon excitation at 450 nm is due to FAD. Excitation at 380 nm resulted in peak emission at approximately 476 nm. This value is between the emission peak at 460 nm of MTHF and at 520 nm of FAD_{ox} (40, 43), indicating the presence of both MTHF and FAD in CryA. Moreover, emission recorded at 520 nm showed a strong peak for excitation at 380 nm. This behavior supports the conclusion that MTHF is bound to CryA and could serve as antenna chromophore transferring excitation energy to FAD as in other cry-DASH and photolyases (3). The action spectrum for photoreactivation in *Phycomyces* wild-type spores shows a peak at approximately 380 nm (34), fully consistent with our results and supporting our proposal of CryA as a folate-binding functional photolyase.

To identify the UV-B photoproducts repaired by CryA, we performed in vitro enzymatic assays with extracts from *E. coli* photolyase-deficient KY1225 cells expressing *Phycomyces* CryA, *E. coli* PHR, or *Arabidopsis* Cry3. CryA extracts repaired T<>T lesions not only in ssDNA and loop-structured duplex DNA but also in dsDNA. The repair of the pyrimidine dimer in dsDNA by CryA was unexpected because cry-DASH are considered as being unable to flip the CPD lesion out of the DNA duplex into the catalytic pocket (19, 20). We did not test whether CryA can repair UV lesions other than T<>T because photolyases are known to be specific for either CPDs or (6, 4)-photoproducts (10). Our search of the *Phycomyces* genome database for proteins of the CPF only resulted in *cryA*. Thus, a gene for (6, 4)-photolyase is not present in the *Phycomyces* genome. Accordingly, (6, 4)-photoproducts must be removed by other repair systems. A candidate for such an activity could be the homolog of Uve1, a yeast protein that removes UV-induced CPDs and (6, 4)-photoproducts. A homolog of Uve1 has been described in *Phycomyces*, and the gene is induced by light (45). Binding constants for damaged and undamaged substrate could not be tested because CryA could not be purified to homogeneity. This circumstance also hampered the determination of catalytic turnover and comparison with those of other CPD-photolyases. A molecular understanding of the mechanism used by CryA to repair CPDs in dsDNA awaits a cocrystal structure of CryA in complex with the substrate.

Our results confirmed that CryA is capable of repairing CPD lesions in duplex DNA and, thus, behaves like canonical CPD photolyases. The ability of cry-DASH to repair CPDs in duplex DNA is under debate. Selby and Sancar (19) analyzed cry-DASH from *A. thaliana* (*At*cry3), *Vibrio cholerae* (*Vc*Cry1) and *Xenopus laevis* (XlCry) and identified their ability to repair CPDs in ssDNA. They also showed that these enzymes were unable to repair similar lesions in dsDNA. Accordingly, they suggested reclassifying DASH-type cryptochromes as "ssDNA photolyases." In contrast, Daiyasu et al. (46) reported that DASH-type cryptochromes from *X. laevis* and zebrafish had some repair activity for CPDs in dsDNA, although less than observed for *E. coli* photolyase. Repair of CPDs in dsDNA like *E. coli* photolyase was described for the DASH-type cryptochrome *Ot*CPF2 from *Ostreococcus tauri* (18). It is possible that these repair activities were the result of an experimental artifact due to the molar excess of enzyme to dsDNA and to the possibility of loop formation near the T<>T. The dsDNA probe that we used in our assays did not allow formation of secondary structures, and our controls with *Arabidopsis* Cry3 showed that our probes could not be repaired by a standard cry-DASH. The cry-DASH from *V. cholerae* (*Vc*Cry1) did not show dsDNA repair activity (19), but Sokolowsky et al. (21) showed that *Vc*Cry1 could dissociate an $oligo(dT)_{10}/oligo(dA)_{10}$ duplex, and monitored the increase in 260 nm absorbance as an indication of T<>T repair in the $oligo(dT)_{10}$ strand. However, an increase in 260 nm absorbance cannot distinguish between DNA strand dissociation and T<>T repair. In conclusion, our results provide the first unambiguous proof to our knowledge for a dsDNA repair activity of a cry-DASH.

Sensory roles have been proposed for fungal CPF members (16, 17, 28, 29), and it is possible that *Phycomyces* CryA plays an additional role as a photoreceptor. However, none of the mutants affected in phototropism have a mutation in *cryA*, arguing against a sensory role for CryA, at least for the phototropism of the fruiting body, and suggesting that the main role of CryA is to act as a photolyase.

Is the photorepair activity of *Phycomyces* CryA an evolutionary step forward, or does it represent an early stage in the evolution of cry-DASH? Fungi possess genes for CDP-photolyases and cry-DASH (3). Our characterization of CPF members in the genomes of *Phycomyces* and other species of the Mucoromycotina revealed the presence and absence of these genes during the evolution of this family of fungi. It is likely that the common ancestor of fungi had genes for both, a CPD-photolyase and a cry-DASH, the latter most likely originated from gene duplication of a CPD-photolyase. This conclusion is fully consistent with recent phylogenetic studies of the CPF, indicating the presence of cry-DASH already in the last common ancestor of all eukaryotes (47). During further evolution in the mucoromycotina lineage, class I CPD-photolyases got lost except in the progenitor of *Umbelopsis ramanniana*, which is an early diverging lineage. This observation also implies that the ancestral cry-DASH had the full spectrum of DNA repair activity, which remained intact in Mucoromycotina perhaps due to evolutionary pressure after loss of class I CPD-photolyase. Furthermore, our results suggest that cry-DASH could not evolve a photoreceptor function in those lineages where evolutionary pressure required maintenance of photolyase function. This conclusion is fully consistent with the fact that none of the *Phycomyces* mutants affected in

light perception show mutations in the *cryA* gene. However, we cannot rule out a subtle role of CryA in phototropism or as light sensor in other responses. In addition, our results suggest that cry-DASH in other members of the Mucoromycotina should show photorepair activity. One cannot completely exclude the possibility that the photorepair activity of *Phycomyces* CryA was obtained after further evolution and selective pressure to regain lost dsDNA repair activity from a standard cry-DASH ancestor. However, the regaining of lost enzyme activities by further mutations is unlikely as has been shown for the glucocorticoid receptor (48). We favor the proposal that *Phycomyces* CryA represents an early stage in the evolution of cry-DASH with full dsDNA repair activity that was retained in the evolution of Mucoromycotina fungi.

Materials and Methods

For the biological materials, gene cloning, expression studies, and repair assays, see *SI Appendix, SI Materials and Methods*.

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