Putative Blue-Light Photoreceptors from *Arabidopsis thaliana* and *Sinapis alba* with a High Degree of Sequence Homology to DNA Photolyase Contain the Two Photolyase Cofactors but Lack DNA Repair Activity

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ABSTRACT: The putative blue-light photoreceptor genes of *Arabidopsis thaliana* and *Sinapis alba* (mustard) are highly homologous to the DNA repair genes encoding DNA photolyases. The photoreceptors from both organisms were overexpressed in *Escherichia coli*, purified, and characterized. The photoreceptors contain two chromophores which were identified as flavin adenine dinucleotide and methenyltetrahydrofolate. This chromophore composition suggests that the blue light photoreceptor may initiate signal transduction by a novel pathway which involves electron transfer. Despite the high degree of sequence identity to and identical chromophore composition with photolyases, neither photoreceptor has any photoreactivating activity.

Near-UV (320−400 nm) and blue light (400−500 nm) induce a variety of responses in microorganisms, fungi, and plants. In plants, the responses known to be mediated by blue light include phototropism, hypocotyl elongation, stomatal opening, and expression of specific genes (Kaufman, 1993; Short & Briggs, 1994; Liscum & Hargarter, 1994). Currently, there is very little biochemical information available on the structure and reaction mechanism of the blue-light photoreceptors [see Hohl et al. (1992a,b) and Short & Briggs (1994)]. However, two recent genetic studies have revealed a remarkable insight into the structure and function of the plant blue-light photoreceptors: it was found that the genes encoding putative blue-light photoreceptor in *Arabidopsis thaliana* (Ahmad & Cashmore, 1993) and in *Sinapis alba* (Batschauer, 1993) have high degrees of sequence identities to the *phr* genes which encode the DNA repair protein DNA photolyase of microbial origin, raising the possibility of evolutionary relationship and pointing to a precise mechanism of signal transduction by the blue-light photoreceptors. Even though current evidence suggest that, in particular, the HY4 gene encodes a blue-light photoreceptor, direct evidence that the corresponding proteins are photoreceptors is lacking [see Briggs (1993)]. Hence, when HY4 (*A. thaliana*) and SA-phr1 (*S. alba*) are mentioned as blue-light photoreceptors, it should be understood that putative blue-light photoreceptor is meant.

Photolyase repairs UV-damaged DNA by photoinduced electron transfer (Sancar, 1994). The enzyme has a $FADH_2$ cofactor and either methenyltetrahydrofolate (MTHF)[1] or 8-hydroxy-5-deazariboflavin (8-HDF) as the "second chromophore". The second chromophore absorbs a photon and transfers energy to $FADH_2$, which in turn transfers an electron to a bound pyrimidine dimer (Pyr⟨⟩Pyr) to initiate bond rearrangement which splits the cyclobutane ring and thus restores the pyrimidines. The second chromophore binds to the NH_2-terminal domain while the carboxy-terminal domain, which is highly conserved, is involved in binding to DNA and to $FADH_2$ (Malhotra et al., 1992). Both HY4 and SA-phr1 have higher degrees of sequence homology to 8-HDF class photolyases, especially in the NH_2-terminal half which is thought to be involved in second chromophore binding. Hence, it was proposed that HY4 and SA-phr1 may contain 8-HDF and $FADH_2$ as chromophores (Ahmad & Cashmore, 1993; Batschauer, 1993). In this paper we describe the overproduction and purification of these two photoreceptors. We found that both photoreceptors contain MTHF and FAD presumably in the two-electron-reduced form. These findings suggest a new mechanism for signal transduction and also raise the possibility that photolyase in addition to its role in DNA repair may function as a blue-light photoreceptor in certain organisms.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *Escherichia coli* strains UNC523F′laci^q (*phr*::*kan uvrA*::*TN10*), UNC3112-[Δ(*Kdp-phr*) Δ(*gal*-bio) *uvrA*::*Tn10*], and NM522 (*hsdR*$^-$ *hsdM*$^-$ *ung*$^+$) were used as host strains for cloning of photoreceptor genes, overproduction, and purification of recombinant proteins. The pMal-c2 plasmid used for gene fusions was obtained from New England Biolabs.

Construction of Maltose Binding Protein (MBP): Photoreceptor (PR) Fusions with HY4 and SA-PHR1. The HY4 gene of *A. thaliana* was isolated from a cDNA library (kindly provided by R. Davis, Stanford University) by PCR using primers which hybridize to the 5′ and 3′ ends of the published sequence (Ahmad & Cashmore, 1993). The nucleotide sequences of the primers were as follows:

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[1] Abbreviations: MTHF, methenyltetrahydrofolate; 8-HDF, 8-hydroxy-5-deaza-riboflavin; Pyr⟨⟩Pyr, cyclobutane pyrimidine dimer; MBP, maltose binding protein.

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PCR1 (33-mer): AAC GGG GAA TTC ATG TCT GGT TCT GTA TCT GGT

PCR2 (36-mer): AAC GGG CTG CAG CCC GGT TTG TGA AAG CCG TCT CCA

PCR3 (corresponding to nt 1510−1530 of HY4: 33-mer which also includes *Pst*I restriction site): AAC GGG CTG CAG, AAG TCC, TTC, TTC, GGA TCC GTT

Amplification reactions were performed in reaction mixtures containing 1 μg of cDNA, 1 μM primers, and 200 μM dNTPs in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 units of Taq polymerase in 100 μL overlayed with 100 μL of mineral oil. The PCR reaction was carried out in a Perkin-Elmer/Cetus DNA Thermal Cycler. The first denaturation was done at 94 °C for 5 min and at 94 °C for 1 min for the subsequent 30 cycles; the annealing was at 55 °C for 2 min, and the extension was at 72 °C for 1.5 min. The last cycle was extended to 10 min to ensure completion of the final extension. By using PCR1 with PCR2 and PCR3, fragments of 1554 and 2067 bp were amplified, extracted with phenol, phenol/chloroform, and precipitated with ethanol. The DNA was resuspended in appropriate buffers, digested with *Eco*RI and *Pst*I, and cloned into the polylinker region of pMal-C2 vector to obtain the plasmid pKM1995 which expresses MBP-HY4 fusion protein expressing the 1554 bp fragment of HY4 which contain the entire *phr*-homology region. The construct (PKM 1995a) containing 2067 bp expresses the entire HY4 sequence including the C-terminal tropomyosin-homology region. The latter construct was poorly overproduced and of low solubility. Therefore, this full-length construct was tested only *in vivo* for complementing the Phr$^-$ phenotype.

To obtain a plasmid expressing the *S. alba* photoreceptor protein in the form of MBP fusion, we subcloned the gene from the SA-phr1pQE-10 plasmid (Batschauer, 1993). Using appropriate PCR primers an *Eco*RI site was created 5′ to the ATG start codon and *Xba*I site 3′ to the termination codon. The amplified fragment was digested with these enzymes and cloned into the polylinker site of pMal-C2 to obtain pKM1996 which overproduces MBP-PHR1/SA.

UV Irradiation and Photoreactivation. The plasmids expressing MBP-HY4 and MBP-PHR1/SA were inserted into *phr*$^-$ cells UNC523 and UNC3112 to test for photoreactivation complementing activity. The plasmid pUNC969 expressing the *E. coli* photolyase gene was used as a positive control. UV irradiation and photoreactivation were carried out as described previously (Husain & Sancar, 1987). Appropriate dilutions of irradiated and photoreactivated cells were plated in duplicate, and the colonies were counted 24 h after plating. The average of at least two experiments were taken to calculate the fractional survival after the photoreactivation treatment.

Preparation of DNA and Dinucleotide T⟨⟩T Substrates. The DNA substrate containing T⟨⟩T was a 40-mer duplex with a T⟨⟩T in the center and was prepared as described previously (Taylor et al., 1987; Svoboda et al., 1993). The oligomer was a kind gift of J.-S. Taylor (Washington University, Saint Louis, MO). The radiolabeled dinucleotide substrate was prepared as follows (Weinfeld et al., 1989): A 50 pmol solution of thymidylyl (3′−5′) thymidine was dephosphorylated by incubating overnight at 37 °C with 20 units of calf alkaline phosphatase. The protein was precipitated at −80 °C by addition of 2.5 volumes of ice cold ethanol. The supernatant was removed, lyopholized, resuspended in 30 μL of water, and incubated at 100 °C for 5 min to remove residual phosphatase activity. The dinucleotide was then labeled with 115 pmol of [γ-^{32}P]ATP in a reaction mixture (30 μL) containing 20 units of T4 polynucleotide kinase at 37 °C for 2 h. The reaction mixture was then incubated at 65 °C for 10 min and loaded onto a 25% polyacrylamide gel. The radiolabeled dinucleotide was located by autoradiography, and the band was cut into small pieces and incubated overnight in 1.0 mL of water at 37 °C. The samples was then spun for 30 min, and supernatant was carefully removed and lyophilized to 350 μL.

To obtain thymine dimer, the radiolabeled dinucleotide was irradiated with 3000 J/m^2 of 254 nm, loaded onto a 25% polyacrylamide gel, and run at 800 V for 10 h. The T⟨⟩T was located by autoradiography, the band was cut into small pieces, and DNA was recovered as described previously.

Photolyase Assays. The photorepair of DNA by restriction site restoration has been described previously (Li *et al.*, 1993). The assay with the dinucleoide T⟨⟩T was conducted as follows: The reaction mixture (200 μL) containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 5% glycerol, 10 μg of BSA, 5 μg of protein, and the T⟨⟩T substrate (5 nM) was exposed to photoreactivating light from Sylvania Black Light (200 ergs mm^{-2} s^{-1}) at 4 °C from 5 to 180 min. The reaction mixtures were then extracted with phenol and phenol/chloroform, and the protein was precipitated with 2.5 volumes of ethanol. The supernatant containing TT and T⟨⟩T was lyophilized to dryness and resuspended in 10 μL of water, and the products were analyzed on a 20% denaturing polyacrylamide gel.

Overproduction and Purification of MBP-HY4 and MBP-PHR1/SA. The fusion proteins were purified by affinity chromatography on an amylose resin. *E. coli* UNC523F′lacI^q/pKM1995 or UNC523F′lacI^q/pKM1996 were grown in Luria broth to A_{600} = 0.6 and induced with 0.1 mM IPTG for 8 h. Cells were harvested and resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 10% sucrose) per liter of culture. After a freeze−thaw cycle, the cells were sonicated 10 × 10 s with a Branson sonicator. The cell debris was removed by centrifugation at 32000*g* for 30 min, followed by a spin at 120000*g* for 60 min. The cell-free extract was dialyzed against buffer B (100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 20% glycerol) + 0.1 M KCl and loaded onto an amylose column equilibrated with the same buffer (30 mL of resin for cell-free extract from 10−15 L of culture). The column was washed with six column volumes of buffer B + 0.1 M KCl, and the bound proteins were eluted with buffer B + 0.1 M KCl + 10 mM maltose. Three milliliter fractions were collected, and the fusion proteins were located by SDS−PAGE and Coomassie staining. Fractions containing the photoreceptor proteins were combined, concentrated by ultrafiltration, and dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 50% glycerol) and stored at −80 °C.

Purification of Cofactors by Thin-Layer Chromatography. The photoreceptors were denatured with 0.8% SDS in 0.1 M HCl at 45 °C for 30 min. The sample was applied to a

Sephadex G-15 column equilibrated with 0.1 M HCl. The fluorescent fractions were identified by 350 nm excitation and 470 nm emission. The samples were lyophilized to dryness, and the two chromophores were separated by TLC in two different solvent systems. Solvent A: *n*-butanol/acetic acid/water, 5:2:3. Solvent B: ethylacetate/methanol/acetic acid, 100:10:1.

Spectroscopy. The absorption spectra were recorded with a Hewlett-Packard Model 8451A spectrophotometer. Fluorescence measurements were made at 4 °C in a Shimadzu RF5000 U spectrofluorometer.

RESULTS

Lack of Photoreactivation Activity in the HY4 and SA-phr1 Photoreceptors. The genes encoding the apoprotein of *A. thaliana* and *S. alba* photoreceptors, HY4 and SA-phr1, respectively, were cloned into the polylinker region of the pMal-c2 vector to obtain in-frame fusions with the *malE* gene encoding maltose binding protein (MBP). The resulting plasmids, pKM1995 and pKM1996 (Figure 1), express the photoreceptors in the form of MBP fusion proteins from a *tac* promoter. These plasmids were inserted into *E. coli* UNC523 which is deleted in the *phr* gene. Because of high sequence homology with the photolyase *phr* gene, we considered the possibility that the photoreceptors may have photolyase activity and therefore tested the constructs for complementing the Phr$^-$ phenotype. Compared to the photoreactivation provided by the *E. coli* photolyase (pUNC 969), the complementation by the plasmids PKM 1995 (HY4) and PKM 1996 (SA-phr1) was marginal or absent (Figure 2). We also tested the plasmid expressing the entire HY4 sequence including the tropomyosin homology region at the carboxy terminus and failed to detect any photoreactivation (Figure 2, square) above control cells (Figure 2, circles). Since a previous study had detected a weak complementing activity with SA-phr1, we considered the possibility that the MBP part of the fusion proteins may interfere with photoreactivation. We conducted three types of experiments to address this point. First, MBP-phr fusion proteins expressing either the *E. coli* or *Bacillus firmus* photolyase were tested for complementation and both were found to complement fully (data not shown). Second, the original SA-phr1 construct (SA-phr1pQE-10) which appeared to marginally complement in another *phr*$^-$ strain (Batschauer, 1993) was tested in UNC523 and failed to complement. Finally, both proteins were purified (see below) and tested with sensitive *in vitro* assays for photolyase activity.

Lack of Photolyase Activity in Vitro. We reasoned that the *in vivo* assay for photoreactivation may not have the sensitivity to detect a weak photolyase activity associated with the photoreceptors. Therefore, the purified proteins were tested *in vitro* with two extremely sensitive assays capable of detecting weak photolyase activity.

In the restriction site restoration assay, radiolabeled DNA with a T⟨⟩T at the *Mse*I site (TTAA) of a duplex is subjected to photoreactivating treatment and then digested with *Mse*I; only the repaired DNA is digested by the enzyme to yield a smaller fragment (Li *et al.*, 1993). Results of this assay are shown in Figure 3. Neither HY4 nor SA-phr1 had any detectable activity. An approximate calculation shows that these proteins cannot have more than 0.01% of the activity associated with the *E. coli* photolyase.

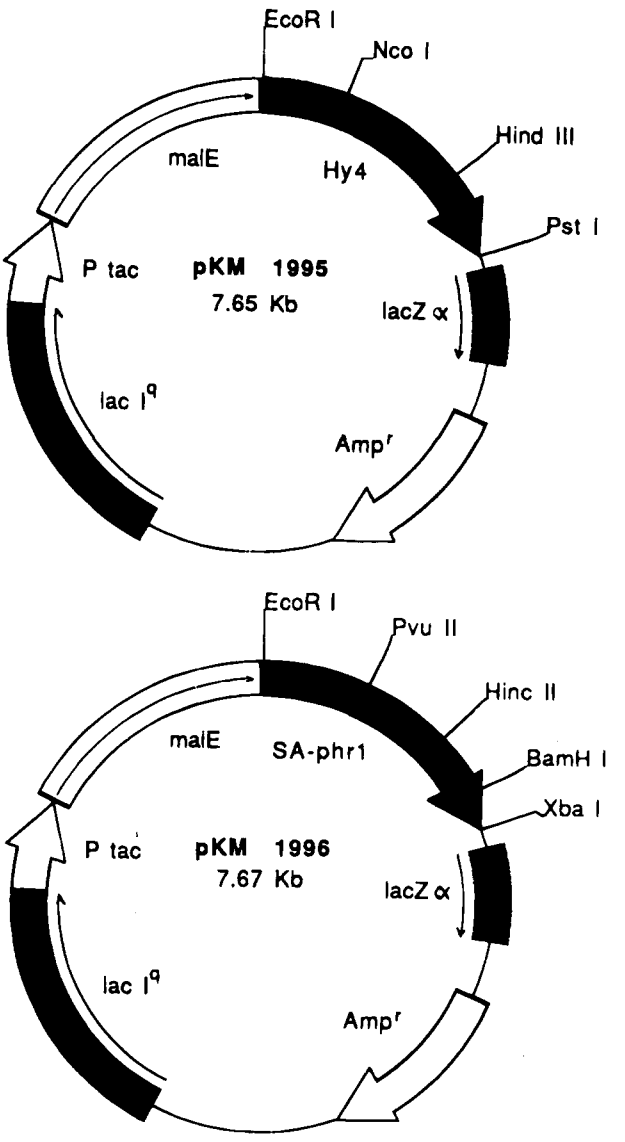

FIGURE 1: Restriction map of plasmid pKM1995 (MBP-HY4) and pKM1996 (MBP-PHR1/SA) which overproduces a blue-light photoreceptor in *E. coli*. The tac expression cartride is indicated by the blank arrow, MBP is indicated by lined blanked arrow, and the direction of transcription of the amp and photoreceptor genes are shown by filled arrows.

The lack of repair by these proteins could be due to lack of binding to DNA, lack of catalysis, or both. In fact, both proteins have a nonconservative substitution of the position corresponding to W277 in *E. coli* photolyase which is necessary for high-specificity DNA binding. Indeed, we did not detect either specific or nonspecific DNA binding with either protein at micromolar concentrations (data not shown). We reasoned that by using T⟨⟩T in a dinucleotide form we would eliminate the requirement of high specificity binding (Kim & Sancar, 1991) since this form would freely diffuse in and out of the active site. Figure 4 shows the results of this assay. Again, within the resolution of the assay no photolyase activity is detectable (specific activity less than 0.01% of *E. coli* photolyase) with either photoreceptor. Thus, we conclude that neither photoreceptor is capable of photolyzing pyrimidine dimers.

Spectroscopic Properties of the Photoreceptors. The photoreceptors were purified by affinity chromatography on amylose. Following elution with maltose, proteins of

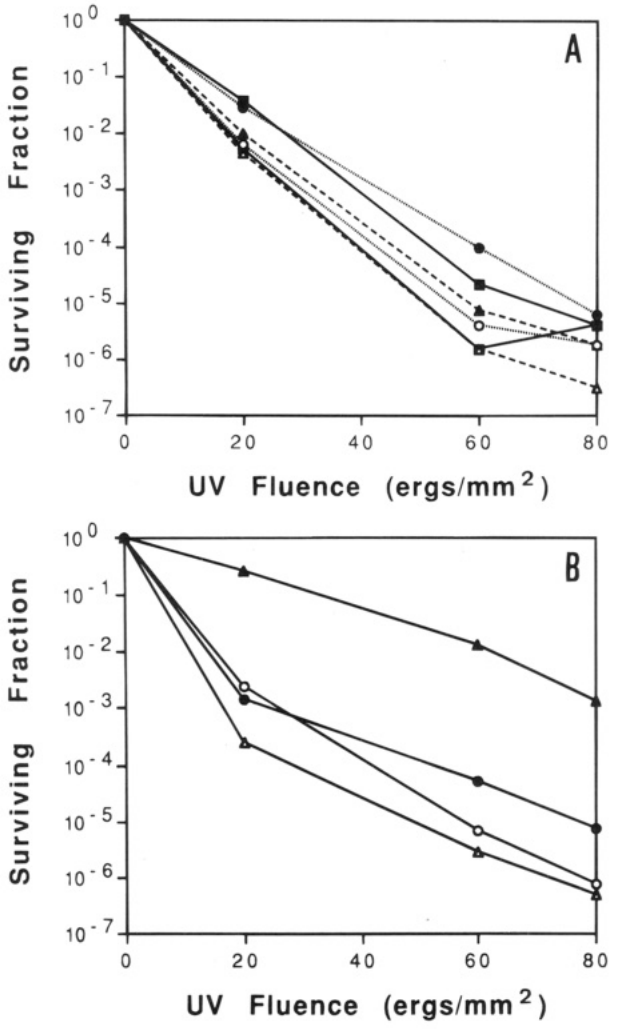

FIGURE 2: UV survival of UNC 523F'lacI^q (phr::kan uvr A::Tn10) containing various plasmids. (A) Open and closed symbols indicate survival before and after photoreactivation, respectively. Square, MBP-HY4; triangle, full length, MBP-HY4 missing the tropomyosin homology region. Circle, MBP. (B) Triangle, pUNC969 (control plasmid expressing *E. coli* photolyase); circle, MBP-PHR1/SA. Cells were grown, plated on LB amp, and irradiated (254 nm) with a GE germicidal lamp. The plates were then exposed to photoreactivating light (black light) for 30 min.

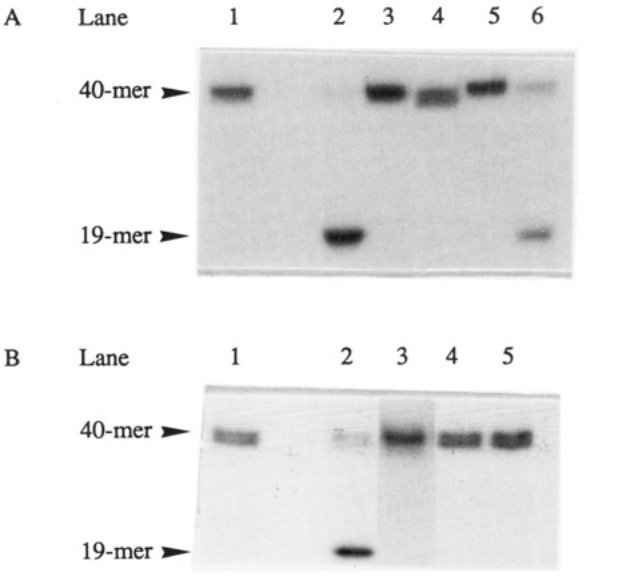

FIGURE 3: Photoenzymatic reversal of T⟨⟩T in DNA by *E. coli* photolyase, but not by photoreceptors of *A. thaliana* and *S. alba*. A 200 μL reaction mixture containing 40-mer substrate with a centrally located T<>T and the indicated concentration of protein were exposed to photoreactivating light for 3 h. The DNA was then incubated at 55 °C for 60 min with 3 μL of 10% SDS and 2 mL of proteinase K (10 mg/mL) and extracted with phenol and phenol/chloroform, precipiated, and digested with *Mse*I, which cuts only repaired DNA. The digested DNA was separated on a 12% denaturing polyacrylamide gels. (Panel A) *S. alba*. Lane 1, no enzyme; lane 2, 1 μg of *E. coli* photolyase; lanes 3−6 contain *S. alba* MBP-PHR1/SA protein; lane 3, 100 μg of cell-free extract of MBP-PHR1/SA overexpressing cells; lanes 4 and 5, 5 and 10 μg, respectively, of purified MBP-PHR1/SA protein; lane 6, cell-free extract of MBP-PHR1/SA overexpressing cells mixed with 1 ng of *E. coli* photolyase. (Panel B) *A. thaliana*. Lane 1, no enzyme; lane 2, 1 μg of *E. coli* photolyase; lane 3, 100 μg of cell-free extract of MBP-HY4 overexpressing cells; lanes 4 and 5, 5 and 10 μg, respectively, of purified MBP-HY4 protein.

expected sizes were obtained at about 80% purity, the contaminants being mostly degradation products which also bind to the amylose column because they contain the MBP part of the fusion protein (Figure 5). At this stage of purification the photoreceptors were of sufficient quality for spectroscopic analysis because control columns with cell-free extract from cells lacking plasmids did not retain any chromogenic material.

Concentrated solutions of both MBP-HY4 and MBP-PHR1/SA have faint yellow color. The absorption and fluorescence spectra of these proteins are shown in Figure 6. The absorption spectra of the two photoreceptors (Figure 6A,C) are nearly identical, with an absorption maximum at 410 nm and residual absorption extending up to 700 nm. These spectra are very similar to that of the photolyase from *B. firmus* which has been shown to contain methenyltetrahydrofolate (MTHF) and FAD neutral radical (Malhotra et al., 1994). Indeed, the fluorescence emission spectra of both photolyases yielded a peak at 460 nm consistent with MTHF emission and a second peak (HY4) or a shoulder (SA-phr1) at 520 nm (Figure 6B,D) consistent with flavin (reduced or fully oxidized) emission. Since folate class photolyases contain FAD in all its three oxidation states in various proportions (Sancar, 1994), we attributed the 520 nm emission to FAD.

To further confirm the identity of the chromophores, the MBP-HY4 fusion protein was investigated in more detail. The protein was denatured by boiling in 0.8% SDS, the precipitate was removed by centrifugation, and the absorption (Figure 7A) and a fluorescence (Figure 7B) spectra of the supernatant were measured. Both are typical of FAD_{ox} with 375 and 450 nm peaks in the absorption spectrum and a 520 nm peak in the emission spectrum. The fluorescence excitation spectrum determined by measuring emission at 510 nm yielded a spectrum similar to the absorption spectrum (Figure 8A). Furthermore, the fluorescence intensity at pH 2.6 was about 8-fold higher than that at pH 7.7 (Figure 8B), thus identifying the flavin as FAD and not FMN or riboflavin (Faeder & Siegel, 1973).

Identification of the Second Chromophore. Figure 6 strongly suggests that both photoreceptor have MTHF because of strong similarity of the absorption and fluorescence spectra to those of other folate class photolyases (Sancar, 1994). However, Figures 7 and 8 provide evidence only for FAD. This can be explained by assuming that upon release from the enzyme MTHF is rapidly converted to nonfluorescent 10-formyltetrahydrofolate with an absorption peak at 260 nm (Johnson et al., 1988). Upon exposure to

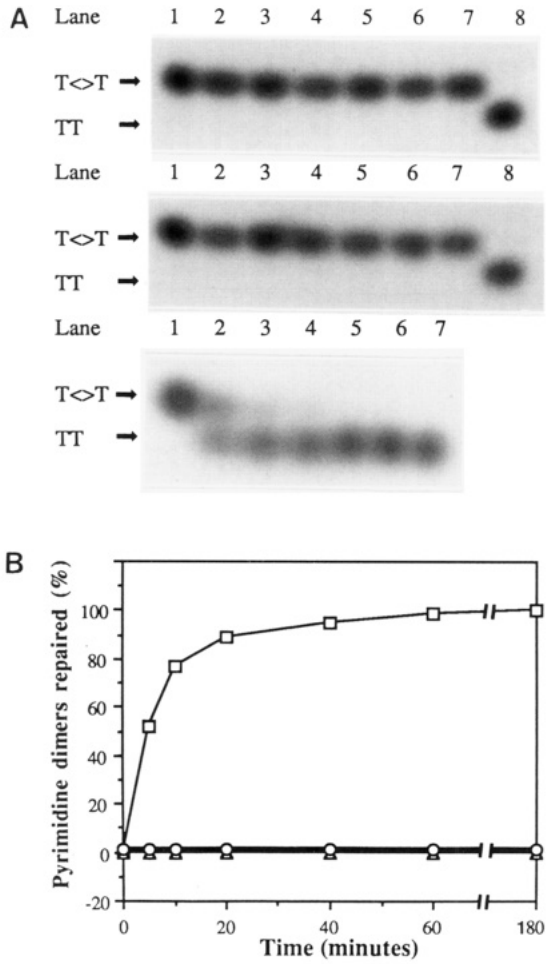

FIGURE 4: Repair of T⟨⟩T in dinucleotide form by photolyase but not photoreceptors. The T⟨⟩T dinucleotide (5 nM) was mixed with 100 nM *E. coli* photolyase or 1 μM photoreceptors and exposed to photoreactivating light (360 nm at 200 ergs mm^{-2} s^{-1}) for the indicated times. (A) Products were analyzed on sequencing gels. Lanes 1−7 correspond to photoreactivation times of 0, 5, 10, 20, 40, 60, and 180 min, respectively. In lane 8, T⟨⟩T plus *E. coli* photolyase mixture was illuminated for 180 min. Top panel, MBP-HY4; middle panel, MBP-PHR1/SA; bottom panel, *E. coli* photolyase. (B) Quantitative analysis of data shown in panel A. Square, *E. coli* photolyase; circle, MBP-PHR1/SA; triangle, MBP-HY4.

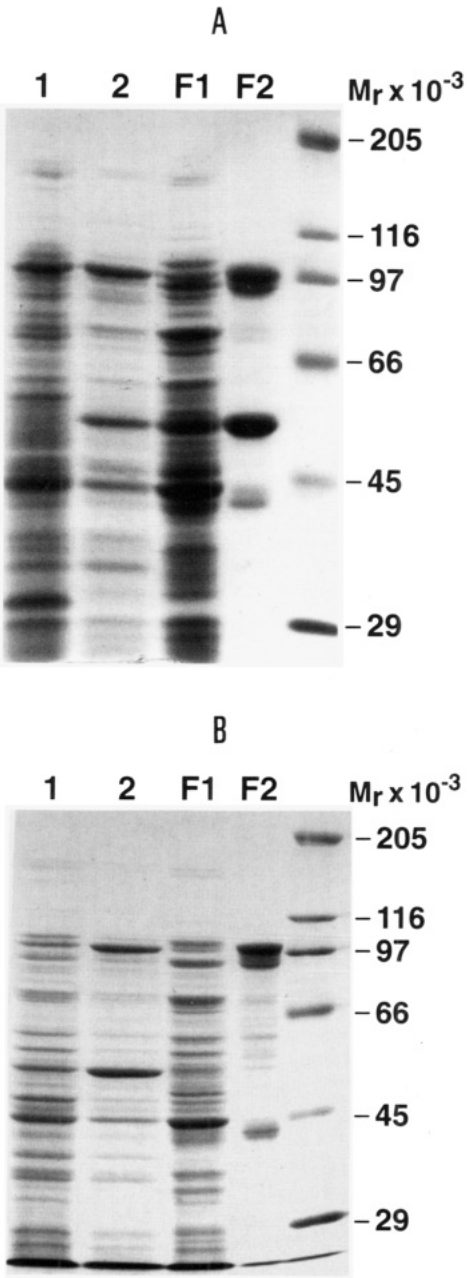

FIGURE 5: Coomassie blue staining of proteins separated by 10% SDS−PAGE during the purification of (A) UNC 523F′lacI^q/pKM 1995 (MBP-HY4) and (B) UNC 523F′lacI^q/pKM 1996 (MBP-PHR1/SA). Lane 1, uninduced cells; lane 2, cells induced with IPTG; lane F1, crude extract; lane F2, peak fraction after amylose column; lane 5, molecular mass marker in kilodaltons.

air this species is oxidized to fluorescent 10-formylfolate. Therefore, to identify the second chromophore the denatured enzyme (in 0.1 M HCl plus 0.8% SDS) was applied to a Sephadex column equilibratd with 0.1 M HCl to separate the cofactors from the apoenzyme (Johnson et al., 1988). The cofactor fractions were identified by fluorescence using excitation at 350 nm. The fractions containing fluorescent material were lyophilized to dryness and the two chromophores were separated by TLC in two different solvent systems (solvent A, *n*-butanol/acetic acid/water, 5:2:3; solvent B, ethylacetate/methanol/acetic acid, 100:10:1). By using authentic FAD as a standard during TLC the band corresponding to the second chromophore derivative was easily located and scraped off the TLC plates. The chromophore was dissolved in 0.1 N HCl to convert the 10-formyl-THF to 5,10-MTHF (Johnson et al., 1988) and analyzed. Figure 9A,B shows that the exciation and emission spectra of the isolated second chromophore are identical to those of authentic MTHF. Thus we conclude that the second chromophore of the HY4 chromophore is MTHF leading us to predict that the SA-phr1 photoreceptor which in native form is very similar to HY4 also contains FAD and MTHF.

DISCUSSION

The *A. thaliana* blue-light photoreceptor gene HY4 was isolated by gene tagging from a population of transgenic lines of *Arabidopsis* containing random T-DNA insertions. One of these insertions yielded a mutant allele of HY4 (hy4-2) which rendered the plant unresponsive to blue light with respect to inhibition of hypocotyl growth. The DNA flanking the insert was cloned and sequenced and then was used as a probe to clone a cDNA which encoded a protein of 681 amino acids (M_r = 75 800). The sequence revealed a remarkable homology with the apoprotein of the DNA repair

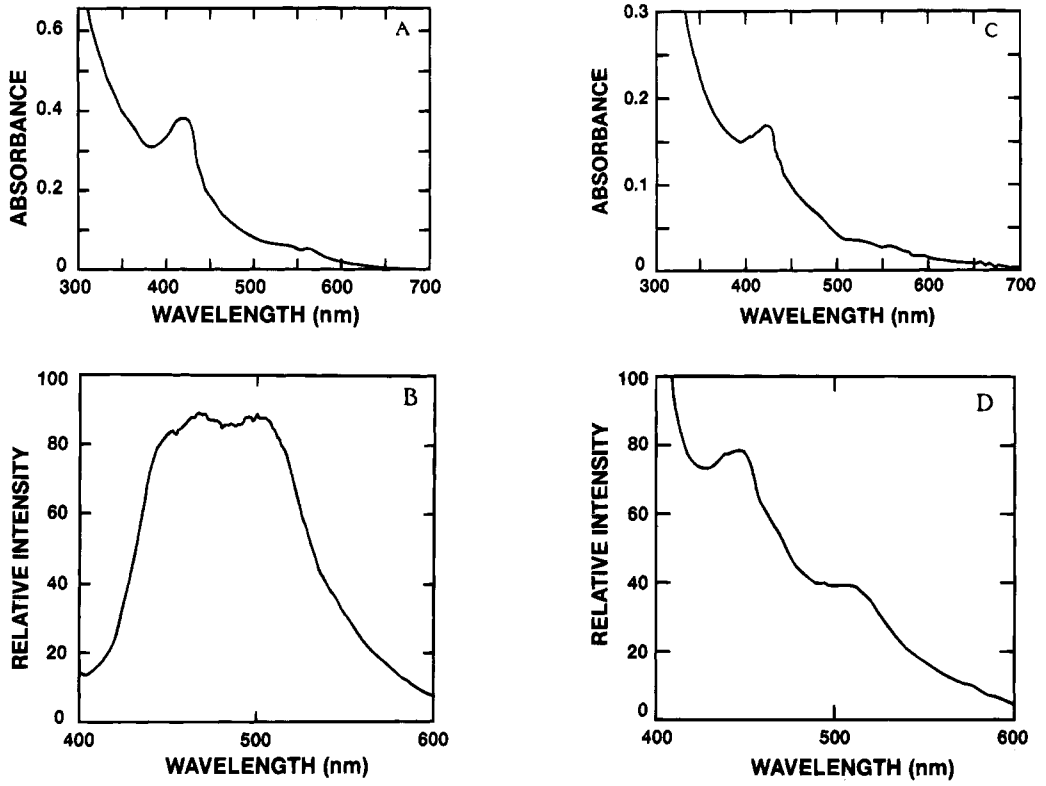

FIGURE 6: Absorption and fluorescence spectra of blue-light photoreceptor fusion protein MBP-HY4: (A) Absorption of E-FADH—Pterin. (B) Fluorescence emission spectra were recorded by monitoring excitation at 390 nm. MBP-PHR1/SA: (C) Absorption spectrum. (D) Fluorescence emisison spectra were recorded by monitoring excitation at 390 nm.

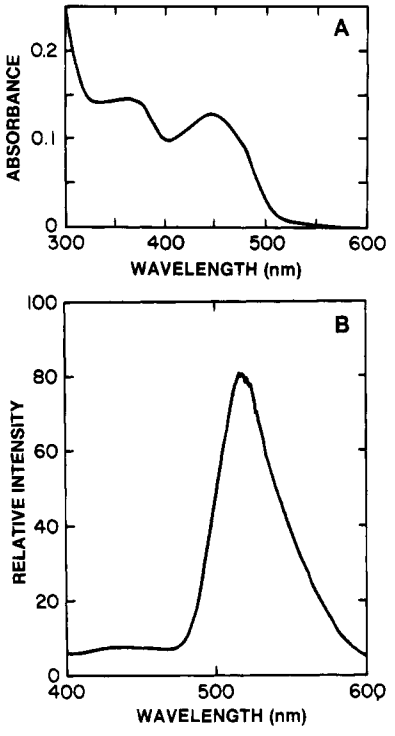

FIGURE 7: Spectroscopic analyses of blue-light photoreceptor fusion protein (MBP-HY4). (A) The protein was denatured by addition of 0.8% SDS, boiled for 5 min, and centrifuged at 15 000 rpm for 10 min, and absorption was taken. (B) Fluorescence emission spectra were measured with excitation at 390 nm.

enzyme DNA photolyase. Over a stretch of 500 N-terminal amino acids HY4 has about 30% sequence identify with all microbial photolyases sequenced to date. In addition, the carboxy-terminal 86 amino acids has 30% identity to the rat smooth-muscle tropomyosin A (Ahmad & Cashmore, 1993).

The mustard (*S. alba*) photoreceptor gene was cloned by using a set of synthetic oligonucleotides with degenerate sequence corresponding to a highly conserved region in the carboxy terminal of microbial photolyases to screen a cDNA library. A cDNA clone (SA-*phr1*) encoding 501 amino acids (M_r = 57 000) was isolated. The amino acid sequence of the putative protein had 18% sequence identity with microbial photolyases with particularly high degree of homology in the carboxy-terminal region spanning amino acids 329–436. SA-phr1 shows 77.6% sequence identity with the consensus sequence of microbial photolyases. It was first thought that SA-*phr1* encoded DNA photolyase because of a small effect it exerted on an *E. coli phr*$^-$ mutant. However, more quantitative *in vivo* studies as well as *in vitro* activity measurements reported in this paper show that it has no photolyase activity, and hence we consider it to be a blue-light photoreceptor of *S. alba*.

From an evolutionary point of view, photolyases pose an interesting problem. Microbial photolyases from prokaryotes, archaebacteria, and unicellular eukaryotes have high level of homology and apparently common origin. In contrast, photolyases from metazoan have very little homology to microbial photolyases even though they appear to have the same chromophore composition and the same reaction mechanism as the folate class microbial photolyases (Kato et al., 1994). These facts may be taken as evidence for convergent evolution. On the other hand, the high degree of sequence homology and the identical chromophore composition between folate class microbial photolyases and blue-light photoreceptors (Ahmad & Cashmore, 1993; Batschauer, 1993) may indicate a divergent evolution of these

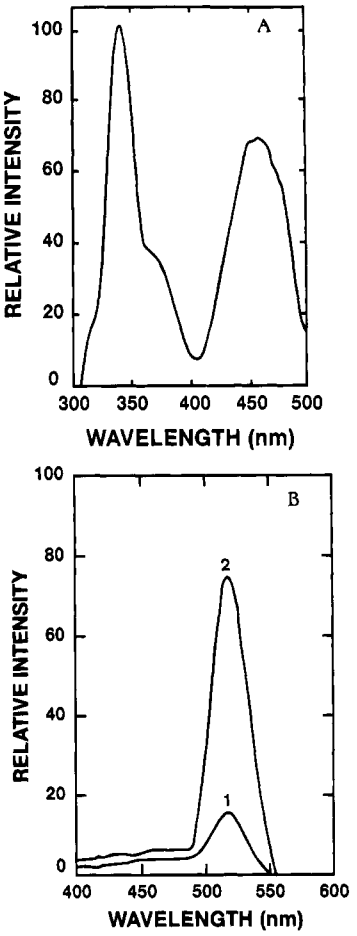

FIGURE 8: Spectroscopic properties of blue-light photoreceptor (MBP-PHR1/SA). (A) Fluorescence excitation spectra were recorded by monitoring emission at 510 nm. (B) Fluorescence emission spectra were measured with excitation at 450 nm at pH 7.7 (curve 1) and at pH 2.6 (curve 2).

two classes of molecules. What the function of the progenitor molecule was is beyond the scope of this work. However, the lack of functional substitution by photoreceptors for photolyase and vice versa raise some questions regarding the structure–function relationship in these important proteins.

In particular, the lack of DNA photolyase activity in the photoreceptors was very surprising to us considering that the HY4 protein shares 60% sequence identity in the region of photolyase active site, including most of the residues known to contact DNA. The fact that the highly conserved W277 (*E. coli*) residue has a nonconservative substitution in HY4 does not explain the lack of photolyase activity. *E. coli* photolyase with nonconservative substitutions at this position has 100–1000-fold lower affinity to damaged DNA, but it binds and repair pyrimidine dimers (Li & Sancar, 1990). In contrast, we have been unable to detect any activity (lower limit about 10^{-5}th the level of *E. coli* photolyase). We reasoned that lack of repair may be due to strong interference by nonconserved residues in HY4 with binding to DNA and that a dinucleotide dimer may diffuse to the active site and be repaired by the photoreceptor. However, repeated attempts failed to reveal any dinucleotide dimer reversal by HY4 under conditions where photoreversal with photolyase was easily detectable (Figure 4).

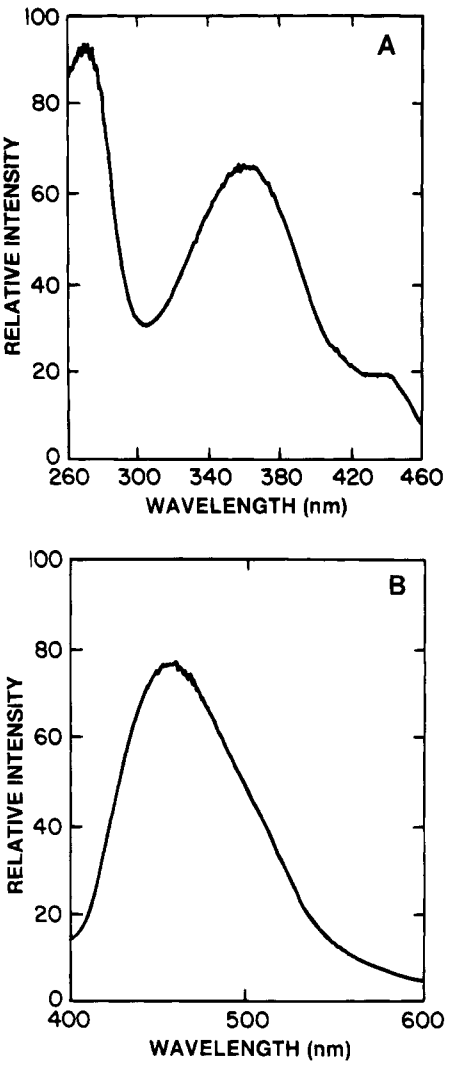

FIGURE 9: Excitation and emission spectra of the second chromophore isolated from blue-light photoreceptor (MBP-HY4) by silica gel thin layer chromatography (solvent system: ethyl acetate/metanol/acetic acid, 100:10:1 by vol). (A) Excitation spectrum was taken at emission wavelength 460 nm. (B) Emission spectrum was taken at excitation wavelength at 350 nm.

The results with the SA-phr1 were equally unexpected, especially since this gene does not have the carboxy-terminal tail of HY4, and in the initial characterization it appeared to confer a marginal photoreactivability onto an *E. coli* strain deleted in the *phr* gene (Batschauer, 1993). However, apparently that "photoreactivation" was nonenzymatic. Indeed, we often observe light-promoted increase in UV survival by the simple presence of plasmid vectors. In line with this observation we have also seen in this study some increase in the number of surviving cells carrying the SA-phr1 construct following photoreactivating light. Compared with the photoreactivation by the *E. coli* photolyase, this increase is probably not significant. Apparently, subtle changes induced by the plasmid on the cell's physiology affect the response to light resulting in ill-defined nonenzymatic photoreactivation effects [see Husain and Sancar (1987)]. More importantly, the purified protein which was soluble and contained both chromophores failed to repair a T⟨⟩T at less than 0.01% the rate of *E. coli* photolyase. Thus, we conclude that SA-phr1 is not photolyase either. Again, testing the enzyme with dinucleotide dimer failed to reveal

any activity. In light of these findings it is tempting to assign SA-phr1 to the photoreceptor class. However, recent immunolocalization studies reveal that most of the protein is in the nucleus (A. Batschauer, unpublished observation). Clearly, further work is needed to clarify the precise function of SA-phr1.

Assuming that SA-phr1 is a photoreceptor, the question arises as to whether or not the photolyases could have dual activities of repair enzymes and photoreceptors. The effector molecules of the blue-light photoreceptors are currently not known. It is possible that photolyase, in addition to binding to DNA can bind to these effector molecules and participate in light-initiated signal transduction.

Finally, the fact that HY4 and SA-phr1 have the same chromophore compositions as the folate class photolyases suggest that these molecules initiate signal transduction by an entirely novel mechanism, that is electron transfer. This is in contrast to all other known signal transduction mechanisms which operate by conformational change, phosphorylation, GTP binding and hydrolysis, and change in quaternary structure.

REFERENCES

Ahmad, M., & Cashmore, A. R. (1993) *Nature 366*, 162−166.
Batschauer, A. (1993) *Plant J. 4*, 705−709.
Faeder, E. J., & Siegel, L. M. (1973) *Anal. Biochem. 53*, 332−336.
Hohl, N., Galland, P., & Saenger, H. (1992a) *Photochem. Photobiol. 55*, 239−245.
Hohl, N., Galland, P., & Saenger, H. (1992b) *Photochem. Photobiol. 55*, 247−255.
Husain, I., & Sancar, A. (1987) *J. Bacteriol. 169*, 2367−2372.
Johnson, J. L., Hamm-Alvarez, S., Payne, G., Sancar, G. B., Rajagopalan, K. V., & Sancar, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2046−2050.
Kato, T., Todo, T., Ayaki, H., Ishizaki, K., Morita, T., Mitra, S., & Ikenaga, M. (1994) *Nucleic Acids Res. 22*, 4119−4124.
Kaufman, L. (1993) *Plant Physiol. 102*, 333−337.
Kim, S. T., & Sancar, A. (1991) *Biochemistry 30*, 8623−8630.
Li, Y. F., & Sancar, A. (1990) *Biochemistry 29*, 5698−5706.
Li, Y. F., Kim, S. T., & Sancar, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4389−4393.
Liscum, E., & Hangarter, R. P. (1994) *Plant, Cell Environ. 17*, 639−648.
Malhotra, K., Baer, M., Li, Y. F., Sancar, G. B., & Sancar, A. (1992) *J. Biol. Chem. 267*, 2909−2914.
Malhotra, K., Kim, S. T., & Sancar, A. (1994) *Biochemistry 33*, 8712−8718.
Sancar, A. (1994) *Biochemistry 33*, 2−9.
Short, T. W., & Briggs, W. R. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 45*, 143−171.
Svoboda, D. L., Smith, C. A., Taylor, J.-S., & Sancar, A. (1993) *J. Biol. Chem. 268*, 10694−10700.
Taylor, J.-S., Garrett, D. S., & Chors, M. P. (1987) *J. Am. Chem. Soc. 109*, 2834−2835.
Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1989) *J. Biol. Chem. 264*, 6364−6370.

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