Purification and Characterization of Three Members of the Photolyase/Cryptochrome Family Blue-light Photoreceptors from Vibrio cholerae*

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The sequence of Vibrio cholerae genome revealed three genes belonging to the photolyase/cryptochrome blue-light photoreceptor family. The proteins encoded by these three genes were purified and characterized. All three proteins contain folate and flavin cofactors and have absorption peaks in the range of 350–500 nm. Only one of the three, VcPhr, is a photolyase specific for cyclobutane pyrimidine dimers. The other two are cryptochromes and were designated VcCry1 and VcCry2, respectively. Mutation of phr abolishes photoreactivation of UV-induced killing, whereas mutations in cry1 and cry2 do not affect photorepair activity. VcCry1 exhibits DNA-binding activity and copurifies with an RNA of 60–70 nucleotides in length.

The photolyase/cryptochrome family encompasses blue-light photoreceptors, which use near UV/blue-light photons as an energy source to either repair far UV (200–300 nm)-induced DNA lesions or to regulate growth and development in plants and synchronize the circadian clock with the daily light-dark cycles in animals (1–3). In addition to their light-dependent functions, both photolyase and cryptochrome also carry out some light-independent functions important for cellular physiology (4, 5); photolyase stimulates excision of UV damage and certain chemical lesions by nucleotide excision repair (4), and cryptochromes inhibit transcription of several circadian clock genes (6–8).

Despite their obvious selective advantages, photolyase and cryptochrome are not universally distributed in nature. Certain organisms, such as Drosophila melanogaster, contain photolyses for both major UV photoproducts, the cyclobutane pyrimidine dimer (Pyr<->Pyr) and the (6-4) photoproduct (2), and a cryptochrome (9, 10), whereas others such as Bacillus subtilis, Haemophilus influenzae, and Caenorhabditis elegans possess neither a photolyase nor a cryptochrome (11, 12). Hence, it is not possible to predict which organisms may or may not contain photolyase/cryptochrome family members based on phylogenetic and evolutionary considerations. In the last decade, genome sequencing projects have identified over 50 photolyase/cryptochrome candidate genes in various organisms including some animal viruses (2). In some instances, these genes have been shown to encode photolyses or cryptochromes using biochemical and genetic approaches. In the majority of cases, the functions of these genes identified by genomics remain to be defined.

We were particularly interested in the photolyase/cryptochrome genes discovered by the Vibrio cholerae sequencing project (13). The sequence of V. cholerae El Tor N16961 revealed three genes belonging to this family. The authors suggested that the presence of three photolyase homologues, more than have been found in other bacterial species sequenced to date, may allow V. cholerae to photorepair the two major UV photoproducts, the cyclobutane pyrimidine dimer and the (6-4) photoproduct, by readily available light energy (13). V. cholerae is an enteropathogen that is transmitted through contaminated water and food, and has a high likelihood of UV exposure during transit. Hence, it would be advantageous if the bacterium had an efficient photorepair system consisting of one or two photolyses for each of the major UV photoproducts. Indeed, it has been shown that, in V. cholerae, photoreactivation plays a much more important role in repair of UV damage compared with other enteropathogenic bacteria such as Escherichia coli (14, 15). Therefore, we decided to investigate the roles of these three photolyase homologues in UV survival of V. cholerae.

Our biochemical analyses show that the proteins encoded by all three genes have the folate and flavin cofactors typical of the photolyase/cryptochrome family. However, genetic and biochemical data indicate that only one is a photolyase, specific for cyclobutane pyrimidine dimer. We designate the corresponding gene and protein Vcphr and VcPhr, respectively. The other two, by convention (16, 17), are designated cryptochromes and are named VcCry1 and VcCry2, respectively. Although cryptochromes are assumed to function as blue-light photoreceptors in organisms ranging from Arabidopsis thaliana (18) to humans (16, 19), there are no photochemical and biochemical data to support the genetic evidence for such a function. In fact, because of a lack of biochemical data on a presumptive “cryptochrome photocycle,” the roles of cryptochromes as photoreceptors, in particular in animals, has been questioned (8).

Currently, the most interesting evidence for a photoreceptor function for cryptochrome is its high sequence homology to photolyase and the presence of both folate and flavin cofactors in all

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characterized cryptochromes (3). However, the native photolyase contains the flavin cofactor in the two-electron reduced FAD$_2$ (or FADH$_2$) form, whereas all cryptochromes analyzed to date contain the flavin in the two-electron oxidized form (16, 20–22). This has led to a model whereby photolyase carries out its regulatory function by an ordinary light-independent flavin oxidoreductase mechanism (23). In fact, even though the active form of flavin in photolyases is FADH$_2$, this cofactor becomes oxidized to either FADH$_2$ or FAD$_{ox}$ inactive forms during purification of most photolases and must be reduced in vitro to activate the enzyme (24). Hence, it is conceivable that the flavin is in the FADH$_2$ form in cryptochromes and that the FAD$_{ox}$ found in isolated cryptochromes is a purification artifact.

In that regard our analyses of the cofactors of VcPhr, VcCry1, and VcCry2 are of special significance. Although VcPhr and VcCry2 do contain FAD$_2$ and FAD$_{ox}$, the purified VcCry1 contains flavin in the two-electron reduced form. We consider this finding the strongest evidence to date that cryptochromes function in a manner analogous to photolases, i.e., by photoinduced electron transfer. Furthermore, VcCry1 under mild purification conditions contains a 60–70-nucleotide RNA. The significance of this latter observation remains to be determined.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—The E. coli strain UN523F lacI6 (pbr-kan uvrA::Tn10) was used as the host strain for expressing the V. cholerae photolyase/cryptochrome proteins (20). The V. cholerae 01 El Tor was obtained from Dr. Fitnat Yildiz (University of California, Santa Cruz, CA). The E. coli pir-116 strain and the plasmid pG704SacB-28 were used for generating null mutants of V. cholerae have been described previously (25, 26). The expression vector pMal-c2 was purchased from New England Biolabs. The antibiotic concentrations used, they were included in culture media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 5 μg/ml; kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 0.5 μg/ml.

**Cloning of V. cholerae phr Homologues into Expression and Gene Disruption Vectors**—The V. cholerae phr homologues were amplified by PCR using primers based on published genomic sequence (13) and V. cholerae 01 El Tor genomic DNA, donated by Dr. Ronald Taylor (Duke University Medical School, Hanover, NH). The homologous sequences on both sides. The resulting PCR clones were digested with the appropriate enzymes and inserted into pMal-c2 (New England Biolabs). The resulting plasmids express the MBP–VcPhr, VcCry1, and VcCry2 proteins. The cloned genes were completely sequenced to ensure that no mutation was introduced during PCR amplification.

The V. cholerae phr homologues were disrupted by inserting a kanamycin$^R$ (kanB) cassette into Vcphr, a chloramphenicol$^R$ (camB) cassette into VcCry1, and a tetracycline$^R$ (tetB) cassette into VcCry2. The kanB, camB, and tetB cassettes were amplified by PCR using pUC8, pNCH40, and pBR322 as templates, respectively. In the case of VcPhr, the gene was disrupted with kanB using overlapping PCR (26). Using the appropriate primers, the kanB gene was flanked by 341 and 350 bp of VcPhr 5′ and 3′ ends, respectively. The construct was then introduced into the suicide vector pG704SacB-28. For VcCry1 and VcCry2, the amplified camB and tetB genes were digested with the appropriate enzymes and inserted into pUNC2002, and pUNC2003, respectively. Then, new PCR primers were used to amplify the antibiotic markers along with 200–300 bp of phr homologous sequences on both sides. The resulting PCR products were digested with the appropriate restriction enzymes and then inserted into the suicide vector pG704SacB-28. The new constructs were named pUNC2004 (VcPhr), pUNC2005 (VcCry1), and pUNC2006 (VcCry2) and used to generate null mutants of the respective genes.

**Generation of Null Mutants of phr Homologues**—Mutants were generated by electroporation of E. coli (pVcphr1-kan) or mating (vccry1 and vccry2::tet). E. coli SM10 containing pUNC2004, -2005, or -2006 was mated with V. cholerae at cell densities of 1 × 10$^8$ cells/ml in LB. The mating mixture was incubated at 37 °C for 3 h with gentle shaking to allow for conjugation. Then, the cells were vortexed to interrupt conjugation, streptomycin was added to the culture to inhibit the growth of E. coli SM10, and the incubation continued for another 2 h. Cells were plated on LB agar containing streptomycin plus kanamycin, chloramphenicol, or tetracycline to select for the exconjugants (25). Colonies were picked from these plates and grown in modified LB agar containing 5% NaCl and 5% sucrose to select for integration of the disrupted genes by homologous recombination (25). Colonies were confirmed from these plates by tested by PCR for disruption of phr homologues using primers of sequences flanking each of the three genes. Gene disruption was confirmed by sequencing the PCR products.

**Purification of V. cholerae Photolyase and Cryptochrome**—To purify VcPhr, VcCry1, and VcCry2, we transferred pUNC2001, pUNC2002, and pUNC2003, respectively, into E. coli UN523. To purify VcPhr, UN523/pUNC2001 was grown in 10-liter LB at 37 °C to A$_{600}$ = 0.6, at which point isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to 0.3 mM and the incubation was continued for an additional 16 h at 22 °C. The cells were harvested by centrifugation, and cell-free extract was prepared by sonication, followed by the removal of cell debris by ultracentrifugation. The cell-free extract was applied to a 10-ml amylose affinity column. Following the extensive washing the protein was eluted with maltose as described by the manufacturer. Fractions containing MBP-VcPhr were identified by SDS-PAGE and Coomassie Blue staining. Fractions containing photolyase were combined and dialyzed against storage buffer that contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 50% (v/v) glycerol. Typical yield was 12 mg of MBP-VcPhr from a 10-liter culture. VcCry1 was purified in a similar manner. However, UN523/pUNC2002 cells were grown at 25 °C before and after IPTG induction. Typical yield for MBP-VcCry1 was 150 mg/10-liter culture. FLAG-VcCry1 was also purified from V. cholerae transformed with pUNC2002-1 by a similar procedure. Initial attempts to purify MBP-VcCry2 under the same conditions were unsuccessful because most of the protein was lost in inclusion bodies. Therefore, UN523/pUNC2003 cells were grown to A$_{600}$ = 0.5 at 37 °C. IPTG was added to 0.3 mM, and incubation was continued at 12 °C for an additional 24 h. The protein was purified by amylase affinity chromatography. Typical yield was 8 mg of MBP-VcCry2 from a 2-liter culture.

**Spectroscopic Analyses**—The absorption and fluorescence spectra were recorded with a Shimadzu UV-1601 spectrophotometer and a Shimadzu RF5000 U spectrofluorometer, respectively. The concentrations of the apoproteins and the cofactors were calculated from their molar extinction coefficients. The theoretical molar extinction coefficients for the three apoproteins at 290 nm, both for the free enzyme and the enzyme bound to its DNA substrate, were calculated by the program of Mildvan et al. (27), and the molar extinction coefficient of FAD$_{ox}$ at 440 nm is 11,300 M$^{-1}$ cm$^{-1}$. The protein concentration was obtained with relative accuracy from the absorption spectrum of the native enzyme. The concentration of MTHF was also calculated from the absorption of the native enzyme at 380 nm. However, there was considerable uncertainty in the molar extinction coefficient of MTHF estimated from this value for VcPhr because the enzyme contains flavin in a mixture of reduced, one-electron, and two-electron oxidized state, and the latter two forms have significant absorbance at 380 nm. This was not a problem for VcCry1 and VcCry2. In VcCry1, essentially all of the flavin is in the two-electron reduced state and does not absorb at 380 nm at a significant level. In VcCry2, MTHF in most preparations is vastly substoichiometric relative to flavin and apoprotein, such that its presence could be ascertained only by florescence spectroscopy. To determine the flavin concentration, the holoproteins were heated at 95 °C for 5 min in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol, and the reduced protein was removed by centrifugation. The absorption spectrum in the 300–700 nm range was recorded, and FAD concentration was calculated from 440 nm absorbance using a molar extinction coefficient of 11,300 M$^{-1}$ cm$^{-1}$. When MTHF is released from the enzyme, the 5–10 methenyl bridge responsible for the 380-nm absorption band is broken at neutral pH to generate flavin-adenine dinucleotide (FAD) and the reduced prosthetic group does not absorb at >300 nm and hence does not contribute to the near UV spectrum of the cofactors (27).

**Photoreactivation**—Wild type V. cholerae N16961 and its mutant derivatives were grown in LB at 37 °C for 16 h. The titers of these...
stationary phase cultures were typically 3–5 × 10^7 cells/ml. Dilutions were made in phosphate-buffered saline and plated in triplicate for each UV or UV plus photoreactivation treatment. General Electric Germicidal lamp G55 emitting mainly at 254 nm was the UV source, and photoreactivation was carried out with a Sylvania F15T8/BLB black light emitting mainly 366 nm. The UV and black light fluences were measured using a UVX digital radiometer (Ultraviolet Products Inc.) fitted with the appropriate sensors. The plates were irradiated with UV fluences of 10, 20, and 40 J m^2 at a fluence rate of 0.5 J m^2 s^-1. For photoreactivation the plates were covered with the plastic cover of the Petri dish and two layers of window glass to filter out radiation below 300 nm. Photoreactivation was carried out for 60 min at a fluence rate of 2.5 J m^2 s^-1, a condition that empirically was determined to produce maximum photoreactivation. Photoreactivation experiments were done under General Electric “gold” fluorescent light to prevent uncontrolled photorepair. Following UV and photoreactivation treatments, the plates were incubated in the dark at 37 °C for 16 h and then the colonies were counted and survivals relative to unirradiated controls were calculated.

**Photolyase Assays—Vibrio cell-free extract was tested for both cyclobutane photolyase and (6-4) photolyase by coupled enzyme assays. In the assay testing for cyclobutane photolyase activity, a radiolabeled substrate containing T<sup>C</sup>T at the MseI restriction site (TTAA), and consequently refractory to cutting by the restriction enzyme, was first treated with the photolyase preparation and then the restriction enzyme. The fraction of the DNA cut by MseI following the photoreactivation treatment is a measure of the extent of photorepair (28).**

**The substrate used for this assay had the following sequence (only top strand is shown), and its preparation has been described previously (16):**

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CTGCCTCATAGTCTAAAGGATCCTCGGCTAATGCTTAAGGTAT(6-4)TATGGACGTGGCCTAGGGCGA-
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**In the assay for (6-4) photolyase activity, we took advantage of the fact that the (6-4) photoproduct inhibits 3’ to 5’ exonuclease digestion by T4 DNA polymerase, and removal of this inhibition by photorepair can be used to measure photoenzymatic activity.** In this assay a 46-bp duplex, which contains radiolabel at the fifth phosphodiester bond 5’ to T6-4/T is used. Repair of the photolysis enables T4 DNA polymerase 3’ to 5’ exonuclease to digest the DNA past the site of the photodamage and release the radiolabel in the form of mononucleotide from which the extent of repair is calculated. The sequence of the duplex used in this assay is as follows (only the lesion-containing strand is shown): CTG

**CGTCATAGTCTAAAGGATCCTCGGCTAATGCTTAAGGTAT(6-4)TATGGACGTGGCCTAGGGCGA-
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**For the photolyase assays, 2 nmol of either substrate was mixed with 100 µg of V. cholerae cell-free extract in reaction buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, and 15% glycerol (v/v). The reaction was either kept in the dark or was exposed to photoreactivating light (filtered through two glass plates) at 4 °C for 90 min. In the case of the cyclobutane photolyase assay, the DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended in restriction enzyme buffer, and digested with 10 units of MseI for 90 min at 37 °C. The reaction products were electrophoresed on 8% polyacrylamide sequencing gel, and the level of repair was determined using a PhosphorImager (Amersham Biosciences). In the case of the (6-4) photolyase assay, following the photoreactivation treatment, the sample was extracted with phenol/chloroform, the aqueous phase was supplemented with 0.1 µg of HaelIII-digested αX174 DNA, 2 units of T4 DNA polymerase was added, the reaction mixture was incubated at 37 °C for 12 min, and the products were separated on a 15% polyacrylamide sequencing gel. The level of repair was determined by the intensity of radiolabeled mononucleotide using a PhosphorImager.

**RESULTS**

**Sequence Analysis of the Three “Photolyase Homologues” of V. cholerae—**The sequences of the proteins encoded by the three phr-like genes of V. cholerae, which we designate VcPhr, VcCry1, and VcCry2 (protein identification code NP_231448), VcCry2 (protein identification code NP_231036) are compared with representative members of the photolyase/cryptochrome family, including a cyclobutane photolyase, a (6-4) photolyase, a plant cryptochrome, and an animal cryptochrome in Fig. 1. Like all other members of this family, the Vibrio proteins exhibit high degree of sequence similarity in the FAD binding domain near the carbonyl terminus. In addition, VcPhr exhibits 53% sequence identity to E. coli photolyase using the GCG Wisconsin Package Program version 10.3, suggesting that this protein is likely to be a cyclobutane photolyase. In contrast, the sequences of VcCry1 and VcCry2 are not that revealing. These proteins exhibit sequence 28% sequence identity to one another and 21–40% identity to VcPhr, E. coli Phr, A. thaliana, and human cryptochromes, and to D. melanogaster (6-4) photolyase. Therefore, genetic and biochemical analyses were necessary to assign functions to these proteins.

**Purification of V. cholerae Photolyase/Cryptochrome Family Proteins—**We cloned the three V. cholerae phr-like genes into the pMal-c2 vector to express the corresponding proteins in E. coli fused with MBP to aid in solubility and purification of the recombinant proteins. All three proteins were expressed at high levels, and VcPhr and VcCry1 were soluble and readily purified. However, VcCry2 was insoluble when cells were grown at >20 °C, therefore, preparative scale quantities could be obtained only when cells were grown at 12 °C, at which the majority of VcCry2 was soluble. Once soluble protein was obtained, all three proteins were readily purified by affinity chromatography on amylose resin. Fig. 2 shows the overexpression and purification of the three proteins as analyzed by SDS-PAGE. As apparent from these figures, after the affinity purification step, the proteins were >95% pure and therefore appropriate for spectroscopic and enzymatic analyses.

**Spectroscopic Properties—**All members of photolyase/cryptochrome family characterized to date contain FAD as an essential cofactor; in addition, these proteins contain a second chromophore, which in the majority of organisms is methenyltetrahydrofolate (MTHF) and in a limited number of species is 5-deazariboflavin (1-3). The folate class enzymes exhibit a major absorption peak at 375–410 nm caused by MTHF and either an additional peak at 440 nm caused by FAD<sub>ox</sub> or several peaks at 480, 580, and 625 nm, caused by flavin blue neutral radical, FADH<sup>+</sup> (3). Often an enzyme preparation can contain a mixture of one- and two-electron oxidized species, giving rise to a complex spectrum in the near UV-visible region (29). The deazaflavin class enzymes exhibit a major peak at 440 nm as a result of deazaflavin and additional peaks in the visible region arising from FAD, as in the case of the folate class enzymes. When a deazaflavin class photolyase/cryptochrome is expressed in E. coli, the absorption spectrum reflects only the contribution of the FAD because E. coli does not synthesize deazaflavin (30, 31).

**Fig. 3** shows the absorption spectra of VcPhr, VcCry1, and VcCry2, respectively. The absorption spectrum of VcPhr exhibited a major peak at 380 nm, minor peaks at 440 and 480 nm, and a tail extending up to 600 nm (Fig. 3A). This spectrum is consistent with the presence of MTHF and a mixture of FADH<sup>+</sup> and FAD<sub>ox</sub> in this protein. The near-UV absorption spectrum of VcCry1 was dominated by a peak at 380 nm and, in this preparation, minor absorption beyond 400 nm ascribable to low levels of oxidized forms of flavin (Fig. 3B). In contrast, the absorption spectrum of VcCry2 showed a peak at 440 nm and a shoulder at 480 nm (Fig. 3C), consistent with a mixture of species containing FAD<sub>ox</sub> and FADH<sup>+</sup>. Although there was a minor shoulder in the 370–390 nm region, from this spectrum alone it cannot be claimed that VcCry2, in this particular preparation, contained MTHF.

Further evidence that all three proteins contain both MTHF and FAD was obtained by fluorescence spectroscopy. Typically, enzyme-bound MTHF has a fluorescence emission maximum at 460–480 nm with an excitation maximum at 360–390 nm (3, 27). Flavin has an emission maximum at 505–520 nm with excitation maxima at 370 nm and 440 nm. However, the flavin excitation spectrum for emission at 520 nm is often complicated in the photolyase/cryptochrome family because of energy trans-
fer from MTHF to FAD when the protein is excited in the 360–390 nm range (32). Fig. 4 shows the fluorescence spectra of VcPhr. When the excitation spectrum was recorded for emission at 460 nm, an essentially symmetrical peak with \( \lambda_{\text{max}} \) at 380 nm was obtained, consistent with MTHF being the predominant chromophore in near-UV in VcPhr. Excitation at 380 nm gave a fluorescence spectrum with a peak at 480 nm and a shoulder at 520 nm, consistent with the presence of both MTHF and FAD (Fig. 4A). When the excitation spectrum was recorded for emission at 520 nm, two peaks at 370 and 440 nm were
obtained, confirming the presence of FAD (Fig. 4B).

Fluorescence spectroscopy of VcCry1 gave results similar to those of VcPhr with some notable differences (Fig. 5). First, the fluorescence emission spectrum with 380 nm excitation had a well defined peak at 460 nm (MTHF) with no discernible shoulder in the 500–520 nm region (FAD), although the emission peak was asymmetric with a tail into long wavelengths from the peak, indicative of contribution from flavin (Fig. 5A). When the excitation spectrum was recorded for emission at 460 nm, an essentially symmetrical peak with $\lambda_{\text{max}} = 380$ nm was obtained, consistent with MTHF being the predominant chromophore in near-UV in VcCry1. In support of this conclusion, excitation of VcCry1 with 440 nm yielded a typical flavin emission spectrum ($\lambda_{\text{max}} = 520$ nm), and when the excitation spectrum was determined for 520 nm emission, a major peak at 380 nm and a minor one at 440 nm were obtained (Fig. 5B) instead of the 370 nm and 440 nm peaks typical of flavin absorbance. It appears that, in VcCry1, MTHF is the main chromophore responsible for excitation of FAD, through Förster resonance energy transfer mechanism, which then decays by fluorescence at 520 nm.

In agreement with the absorption spectrum, the fluorescence spectra of VcCry2 was dominated by flavin (Fig. 6). Excitation at 380 nm exhibited a typical flavin emission at 520 nm (Fig. 6B). Finally, excitation spectrum for emission at 520 nm gave a characteristic flavin absorption spectrum, albeit with a somewhat distorted ratio of 370 nm/470 nm (Fig. 6C), most likely caused by higher contribution of scattering at lower wavelengths in this protein preparation. In summary, the spectroscopic data, combined with the vast literature on the behavior of folate class photolyases, lead us to conclude that all three photolyase-like proteins of *V. cholerae* contain FAD and MTHF as chromophore/cofactors. It must be noted, however, that the three proteins exhibited significantly different spectroscopic properties. This is mainly the result of the different stoichiometries of MTHF in the three proteins and, to lesser degree, of the differences in the microenvironment of FAD in each protein, as evidenced by the different propensities of the flavin to oxidation in the three proteins during purification.

To calculate the stoichiometries of the two cofactors relative to the apoenzyme, the proteins were heat-denatured at neutral pH, and, following removal of the denatured protein by centrifugation, the absorption spectra were recorded. Upon release from the protein, the 5–10 methenyl bridge of the MTHF is broken and the resulting 10-formyltetrahydofolate no longer absorbs at $\lambda > 300$ nm (27). Hence, the absorption spectra of the denatured enzymes in the 300–600 nm range yields typical flavin absorption spectrum (Fig. 7) from which the FAD con-
centration is calculated. The stoichiometry of FAD to apoenzyme is estimated from the ratio of the 440 nm absorption of the denatured enzyme to the absorption of the holoenzyme at 280 nm. The concentration of the MTHF is estimated from the absorption at 380 nm of the holoenzyme. There are some uncertainties in all these estimates. In particular, the calculation of MTHF concentration requires correction for the contribution of flavin at 380 nm, which is dependent on the oxidation state of the flavin. Moreover, the stoichiometries of the cofactors were found to depend on the growth conditions of the cultures and the number of steps in the purification procedure. The range of values found for the three proteins were (FAD:MTHF:apoenzyme): 0.2–1.0:0.2:1.0 for VcPhr, 0.5–1.0:0.5–1.0:1.0 for VcCry1, and 0.1:0.02–0.2:1.0 for VcCry2. The following general trend was observed; VcPhr usually had equimolar flavin and folate, VcCry1 bound the MTHF cofactor more tightly, and VcCry2 bound the FAD more tightly.

The Three Ligands of VcCry1—Of all three Phr-like proteins in V. cholerae, VcCry1 exhibited some unique properties that might be relevant to the reaction mechanisms of cryptochromes in general. In particular, of all cryptochromes analyzed to date, VcCry1 is the only one identified thus far that retains its flavin in the two-electron reduced form as shown clearly in Fig. 8. When the enzyme was purified over a period of 1–2 days either from E. coli (Fig. 8A) or V. cholerae (Fig. 8B), the absorption spectrum exhibited only the 380 nm peak typical of MTHF, even when purification was carried out under aerobic conditions (Fig. 8, solid line) with no significant absorption at $\lambda > 500$ nm. Upon denaturation, the methenyl bond of MTHF is broken to generate 10-formyltetrahydrofolate and the released flavin is oxidized to reveal the typical FAD absorption spectrum (Fig. 8, dashed line). From the 380 nm absorption of the native protein and the 440 nm absorption of the denatured enzyme, it was calculated that in this particular preparation two cofactors were present in nearly 1-to-1 stoichiometry in the holoenzyme. Second, after the affinity purification step, VcCry1 always exhibited a 260 nm absorption peak in the far UV, suggesting the presence of a nucleic acid in the enzyme preparation (Fig. 9A). Indeed, passage of the affinity column-purified protein through a DEAE anion exchange column shifted the far UV absorption maximum from 260 to 280 nm, indicating that the nucleic acid in VcCry1 was loosely bound to the protein. From the absorption of the enzyme at 260 and 280 nm before and after removal of the associated nucleic acid and the known extinction coefficients of the apoprotein and nucleobases (assuming single-stranded nucleic acid), we calculated that a nucleic acid of $\approx 70$ nucleotides is associated with VcCry1. The nucleic acid bound to the enzyme was extracted from the protein by phenol/chloroform and treated with either RNase A or DNase I. As seen in Fig. 9B, the VcCry1-associated nucleic acid is digested by RNase but not by DNase and therefore is identified as RNA. Moreover, from the DNA size markers used in this figure, it appears that the RNA is $\approx 60$ nucleotides in length, in a reasonable agreement with the estimate made from the absorption spectra. The size estimate must be considered approximate, however, until the identity of the RNA is determined.

Functional Assays of V. cholerae Photolyase-like Proteins—The data presented so far show that all three photolyase ho-
mologues of *V. cholerae* contain the two cofactor/chromophores found in the majority of photolyases. To find out whether all three proteins functioned as photolyases as originally suggested (13), we generated strains mutated in one or more of these homologues and tested them for photoreactivation. As shown in Fig. 10A, insertional inactivation of Vcphr abolished photoreactivation completely, suggesting that, of the three phr-like genes, only this one encodes a photolyase. In agreement with this conclusion, inactivation of Vccl and Vcct2 or of both had no effect on the photoreactivation of the mutant strains (Fig. 10B). Thus, we conclude that Vcphr encodes a photolyase and that Vccl and Vcct2, by definition (3, 33) encode cryptochromes. An interesting observation in Fig. 10A is that wild type *V. cholerae* is more UV-resistant than the...

Fig. 6. Fluorescence spectra of VcCry2. A, emission spectrum recorded with 380 nm excitation wavelength. Note that the spectrum is dominated by flavin emission at 520 nm, with a minor folate peak in the 430–450 nm region. B, emission spectrum obtained with 440 nm excitation is characteristic of flavin fluorescence. C, excitation spectrum for emission at 520 nm matches the flavin absorption spectrum.

Fig. 7. Absorption spectra of the chromophores released from *V. cholerae* photolyase homologues by heat denaturation. A, VcPhr; B, VcCry1; C, VcCry2. The proteins were heat-denatured at neutral pH, and, following removal of denatured protein by centrifugation, the absorption spectra were recorded. Upon release from the protein, MTHF is converted to 10-formyltetrahydrofolate, which no longer absorbs in the near-UV (26); hence, the spectra of denatured proteins are those of flavin only.
VcPhr mutant, even in the absence of photoreactivating light. In fact, this is consistent with the "dark function" of photolyase, as has been observed in E. coli and S. cerevisiae, and is the result of the stimulation of nucleotide excision repair by photolyase bound to UV photoproducts (4).

The finding that, at a dose of 10 J/m², where cyclobutane pyrimidine dimers constitute more than 90% of the UV photoproducts (34), photoreactivation completely eliminates the lethal photolesions in wild type but not in the VcPhr mutant (Fig. 10) suggests that VcPhr is a cyclobutane photolyase. This conclusion was confirmed by carrying out in vitro photoreactivation assays with V. cholerae cell-free extracts. As seen in Fig. 11A, cell-free extract from wild type V. cholerae reversed T<>T in a light-dependent reaction (lane 5), whereas extract from the VcPhr mutant did not (lane 3), indicating that V. cholerae has a single cyclobutane photolyase, which is encoded by the gene for VcPhr.

Because (6-4) photoproducts constitute a small fraction of the UV/lesions at low UV dose and ~20% of total lesions even at the highest UV dose used for in vivo photoreactivation experiments (Fig. 10), we were concerned that the photoreversal of these lesions may have escaped detection by the in vivo assay. To address this concern, we performed (6-4) photolyase assays with Vibrio cell-free extract. The results (Fig. 11B) indicate that V. cholerae lacks (6-4) photolyase activity in agreement with the in vivo data that elimination of cyclobutane photolysis by Vephr mutation totally abolished biological photoreactivation in this organism. Finally, when the purified VcPhr, VcCry1, and VcCry2 proteins were tested in vitro, VcPhr behaved identically to E. coli cyclobutane photolyase, whereas VcCry1 and VcCry2 lacked any DNA repair activity (data not shown). However, we consider the data from the biological photoreactivation assay (Fig. 10) and the photolyase assay with

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FIG. 8. Absorption spectra of VcCry1 purified from E. coli or V. cholerae. A, absorption of MBP-VcCry1 purified from E. coli before denaturation (solid line) and after denaturation (dashed line). From the absorbance values of the native enzyme at 380 nm and of the released flavin in the denatured enzyme at 440 nm, a stoichiometry of MTHF:FAD of 1.2:1 is calculated. B, absorption spectrum of FLAG-VcCry1 purified from V. cholerae.

FIG. 9. The RNA ligand of VcCry1. A, absorption spectrum of VcCry1 showing a 260 nm peak (solid line) after purification through amylose resin and a 280 nm peak (dashed line) following passage of the affinity-purified material through a DEAE column. B, analysis of VcCry1-associated nucleic acid. The nucleic acid was extracted from VcCry1 by phenol/chloroform, treated with RNase A or DNase I where indicated, separated on 2% agarose gel, and stained with ethidium bromide. Lane 1, DNA size markers (length indicated in bp); lane 2, VcCry1-associated nucleic acid; lane 3, the nucleic acid treated with RNase A; lane 4, the sample treated with DNase I; lane 5, a 60-nucleotide DNA oligomer treated with DNase I; lane 6, the DNA oligomer used in lane 5.
Photolyase and Cryptochromes in V. cholerae

Fig. 10. Effects of VcPhr, VcCry1, and VcCry2 on UV killing and photoreactivation of V. cholerae. Appropriate dilutions of stationary phase cultures were plated on LB agar and irradiated with 250 nm. The plates were then either kept in darkness or exposed to photoreactivating light (λmax = 366 nm) for 60 min at 22 °C. The plates were then incubated at 37 °C for 16 h, after which colonies were counted and relative survival was determined. A, role of VcPhr. Closed and open circles represent wild type cells without and with photoreactivation; closed and open triangles indicate Vcphr::kan mutant without and with photoreactivation, respectively. The bars indicate standard errors of three independent experiments. B, roles of VcCry1 and VcCry2 in survival. Closed symbols indicate cells kept in darkness; open symbols indicate cells exposed to photoreactivating light. Circles, Vcry1::cam; triangles, Vcry2::tet; squares, Vcry1::cam Vcry2::tet. Bars indicate standard errors of three independent experiments.

Note that there is no statistically significant difference between the survivals of the photoreactivated wild type in panel A and those Vcry mutants in panel B.

cell-free extracts (Fig. 11) more compelling regarding the activities of these three proteins because photolyases expressed in heterologous systems are often inactive (3). In conclusion, the data in this paper considered in its entirety lead us to conclude that, of the three phr-like genes in V. cholerae, only one encodes a cyclobutane photolyase. The other two encode blue-light photoreceptors of unknown functions. In agreement with the general criteria used in naming members of this family of proteins, we have designated the three phr-like genes of V. cholerae Vcphr, Vccry1, and Vccry2, respectively.

DISCUSSION

Photolyase/cryptochrome family proteins mediate light-dependent repair of UV damage in many organisms and a variety of near UV-blue light responses such as growth and differentiation in plants and circadian photoreception in plants and animals (1–3, 35). In addition to their roles in photorepair and phototransduction, these proteins also perform light-independent functions. Photolyase stimulates excision repair of cyclobutane pyrimidine dimers (4), and cryptochrome participates in the transcriptional feedback loop that entrains the molecular clock (3, 8). The structures and functions of photolyases have been studied in some detail (3). In contrast, there is very limited information about the reaction mechanism of cryptochromes. In particular, the primary photochemical reaction carried out by cryptochromes is currently unknown. It has been argued that cryptochromes, which may function as photoreceptors in plants, are simply a component of the signal transduction pathway in photic responses of the circadian clock system in animals (8). It has also been suggested that, although mammalian chromophores have lost their photoreceptive role, they have retained their FAD cofactor so that their transcriptional activity might be regulated by the redox status of the cell (23). The difficulty in obtaining cryptochromes of plant and mammalian origin in their native states (17, 20, 36) has made it problematic to test the various models for cryptochrome function in vitro. Hence, the discovery of cryptochrome in bacteria (Refs. 22, 32, and 37; this work) provides an opportunity to study its role in species more amenable to biochemical analyses. We believe the data presented in this study provide significant insight into the structure and function of cryptochromes. Our main findings and their implications to the reaction mechanisms of cryptochromes in general are briefly recapitulated below.

Cryptochrome/Chromophore Cofactors—VcCry1 contains stoichiometric amounts of FAD and MTHF in most preparations. The significance of this finding is discussed below.

Oxidation States of FAD—In all isolated cryptochromes, from a variety of organisms ranging from A. thaliana (17, 20) to humans (16) to cyanobacteria (22), the flavin is in two-electron oxidized form. The active form of flavin in photolyase is the two-electron reduced (FADH\textsuperscript{-} or FADH\textsubscript{2}) form (27, 38, 39). For
that reason, the question arose as to whether photolyases and cryptochromes use the flavin for different mechanisms (23). Currently, there is no in vitro assay for cryptochromes, making it impossible to directly answer this question. Therefore, our finding that VcCry1 contains the flavin in the two-electron reduced form is strong evidence that all other native cryptochromes also contain the flavin in this form. We suggest that cryptochrome functions in a manner similar to photolyase, i.e. by photoinduced electron transfer to a substrate. In this regard, it should be noted that, of the approximately dozen photolyases isolated and characterized to date, only the S. cerevisiae photolyase retains its flavin in the FADH+ form after purification (40). All other photolyases are known to contain the flavin in this form in vivo (38) and are only active in this form in vitro (see Ref. 3). Indeed, the discovery of the two-electron reduced form of flavin as the active form of the cofactor (24, 38, 41) played a crucial role in elaboration of a unified reaction mechanism for all photolyases (42). Consequently, the finding that VcCry1 contains a two-electron reduced FAD may be the first evidence that all cryptochromes may contain their flavin in this form and mediate photoinduced electron transfer reactions rather than ground-state flavin oxidation-reduction reactions.

The Second Chromophore—Methenyltetrahydrofolate and 5-deazariboflavin are referred to as second chromophores in photolyases because even though under limiting light conditions they may enhance the reaction rate 10−100-fold, they are not essential for enzymatic activity (32, 42). The second chromophore in VcCry1 and VcCry2 is MTHF, as it is for all other plant and animal cryptochromes characterized to date (3). Significantly, VcCry1 is the first cryptochrome that contains stoichiometric MTHF after purification, which lends support to the previous reports on the presence of MTHF in plant and animal cryptochromes. We note, however, that our identification of the second chromophore in VcCry1 as MTHF is based on its spectroscopic and chemical properties. For formal proof, mass spectrometric analyses of the isolated co-factor is needed.

In addition to V. cholerae, cryptochrome has so far been reported in only one other bacterium, the photosynthetic cyanobacterium Synechocystis sp. PCC6803 (22, 37, 43). Synechocystis contains two members of the photolyase/cryptochrome family, which were named PhrA and PhrB, respectively (44). When purified from its native source PhrA contains deazaflavin as a second chromophore and it is a cyclobutane photolyase (44). PhrB was purified as a recombinant protein expressed in E. coli, and it was reported that this protein, which has 38% sequence identity with VcCry1, may represent a new branch of the photolyase/cryptochrome family that do not have a second chromophore, based on the following arguments (43). First, it was suggested that, in Synechocystis PCC6803 PhrB, neither the residues contacting the folate in the x-ray structure of E. coli photolyase nor the residues contacting the deazaflavin in the A. nidulans (Synechococcus 6301) photolyase were conserved (37). Second, the purified PhrB protein lacked the 440 nm absorption peak typical of deazaflavin but exhibited a major peak in the 380−390 nm region that was not assigned to a particular chromophore, even though the protein was shown to contain FAD (22). Finally, the crystal structure of Synechocystis 6803 PhrB obtained by molecular replacement contained FAD but no second chromophore, leading to the conclusion that, in this class of cryptochromes, the second chromophore
may be distinct from folate and deazaflavin or absent altogether (43). Our results clearly show that VcCry1 does have a folate as the second chromophore and strongly suggest that all other members of this branch of the photolyase/cryptochrome family have second chromophores either in the form of folate or deazaflavin. The PhrB protein of Synechocystis 6803 that was used for spectroscopic analysis (22) and for crystallography (43) was made in E. coli, which does not synthesize deazaflavin. In fact, when deazaflavin class photolyses are expressed in E. coli, they contain only the FAD cofactor (30, 31). The FAD-containing protein can be readily supplemented with 5-deazaflavin to obtain holozyme (31). Hence, we strongly suspect that PhrB of Synechocystis 6803 is a deazaflavin-containing cryptochrome, as has been shown for the photolyase encoded by the phrA gene from the same organism (43).

RNA Associated with VcCry1

When we purify MBP-VcCry1 by amylose affinity chromatography, the protein is invariably associated with RNA. Of the 13 photolyase/cryptochrome family members purified in our laboratory by this method, VcCry1 is the only one found to be associated with RNA. The RNA appears to be 60–70 nucleotides in length. At present we do not know whether this is a unique RNA species or multiple species of the same approximate length bound to the cryptochrome. Clearly, further characterization of the RNA is needed to answer this and related questions. It must be noted, however, that of the three ligands (FAD, folate, and RNA) bound to VcCry1, RNA is the one that dissociates most readily. A passage through an anion exchange column under moderate ionic strength removes the RNA without significantly affecting the other two ligands. Hence, it is possible that, although folate and FAD function as chromophore/cofactors in VcCry1, RNA is actually a substrate. In preliminary experiments, however, we have not observed any effect of light on the VcCry1-RNA complex. Similarly, the low amount of VcCry1 currently available from V. cholerae has so far precluded determining whether VcCry1 purified from its native host is associated with RNA.

Role of Cryptochrome in V. cholerae

V. cholerae is an enteric bacterium and therefore protected from light in its host. However, when it is in its natural aquatic environment or is being transmitted through water and foodstuff to its host, it may be exposed to sunlight, which would impart both the harmful UV light and the photoreactivating near UV-visible light. It is notable that V. cholerae relies more heavily on photolyase-mediated photorepair of UV damage than other enteric bacteria such as E. coli (15). As a result, under the experimental conditions shown in Fig. 10, it is rather difficult to observe photoreactivation in E. coli because, under these conditions, the UV photoproducts are eliminated by nucleotide excision repair and postreplication repair quite efficiently. The genome sequence of V. cholerae reveals that, although the organism does possess these repair systems (13), apparently they are not as efficient in preventing cellular death by UV as they are in E. coli. Thus, it is likely that photolyase plays an important role in survival of the V. cholerae species in nature.

Whether or not the Vibrio cryptochromes play a light-dependent or a light-independent role in its survival remains to be determined. Under the experimental system used in our study, VcCry1 and VcCry2 have no effect on the survival of this organism. However, the assay system used is rather artificial, with a UV dose delivered in seconds followed by photoreactivating light delivered over a 60-min period. It is conceivable that, with a different UV light exposure regime, the survival benefit of VcCry1 and VcCry2 would manifest itself. How cryptochromes may confer such a selective advantage is an interesting question. We have found no evidence that they do so by aiding photorepair. In contrast, it was reported that in Synechocystis 6803, even though the protein encoded by phrA was responsible for most of the photoreactivation activity, the phrB mutant also exhibited diminished photoreactivation of UV killing (37). It was suggested that Synechocystis PhrB may function as a (6–4) DNA photolyase or cryptochrome. Synechocystis PhrB exhibits ~8% sequence identity to VcCry1, leading us to believe that the results obtained with VcCry1 would be applicable to the Synechocystis 6803 PhrB as well. Because of the fact that mutation of Vephr eliminated all cyclobutane pyrimidine dimer activity in this organism, and because V. cholerae lacks (6–4) photolyase activity altogether (Fig. 11B), we suggest that both the Vibrio protein and its Synechocystis equivalent are cryptochromes. How cryptochrome might contribute to light-dependent UV survival in Synechocystis is not clear at present, but our data indicate that it is not through a direct repair function of the PhrB cryptochrome. We have considered the possibility that VcCry1 and perhaps other bacterial cryptochromes may aid survival by mediating phototaxis and enabling the bacteria to swim away from the harmful sunlight. Even though V. cholerae appears to have a well developed chemotaxis system (13), there are no reports on phototaxis in V. cholerae; hence, this conjecture needs experimental testing and verification.

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