The Role of Conserved Amino Acids in Substrate Binding and Discrimination by Photolyase*

Mark E. Baer and Gwendolyn B. Sancar‡

From the Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7260

DNA photolyases catalyze the light-dependent repair of pyrimidine dimers in DNA. We have utilized chemical modification and site-directed mutagenesis to probe the interactions involved in substrate recognition by the yeast photolyase Phr1. Lys617 was protected from reductive methylation in the presence of substrate, but not in its absence, and the specific and non-specific association constants for substrate binding by Phr1(Lys617 → Ala) were decreased 10-fold. These results establish a role for Lys617 in substrate binding. Mutations at Arg69, Lys642, and Trp387 reduced both the overall affinity for substrate and substrate discrimination. Sites of altered interactions in ES complexes were identified by methylation and ethylation interferences techniques. Interaction with the base immediately 3' to the dimer was altered in the Phr1(Lys617 → Ala)-DNA complex, whereas interactions with the phosphate and base immediately 5' to the dimer were reduced when Phr1(Arg69 → Ala) bound substrate. Multiple interactions 5' and 3' to the dimer were perturbed in complexes containing Phr1(Trp387 → Ala) or Phr1(Lys642 → Ala). In addition the quantum yield for dimer photolysis by Phr1(Trp387 → Ala) was reduced 3-fold. The locations of these mutations establish that a portion of the DNA binding domain is comprised of residues in the highly conserved carboxyl-terminal half of the enzyme.

DNA photolyases catalyze the light-dependent repair of pyrimidine dimers in DNA (for a recent review see Sancar, 1990). The enzyme binds to substrate, and subsequent absorption of a photon of near UV or visible light (300-500 nm) provides energy to drive electron transfer from enzyme-bound FADH₂ to the dimer, thereby initiating a cycloreversion reaction which restores the photolyzed monomer. Enzymatic photoreactivation has been detected in numerous organisms, including fungi, bacteria, plants, invertebrates, and vertebrates, and during the last 10 years genes encoding photolyase apoenzymes from both prokaryotes and eukaryotes have been cloned and sequenced (Sancar, 1990; Yasuhira and Yasui, 1990). Seven of the eight cloned genes are from microorganisms (Escherichia coli, Salmonella typhimurium, Anacystis nidulans, Halobacterium halobium, Streptomyces griseus, Saccharomyces cerevisiae, and Neurospora crassa) and comparison of the amino acid sequences predicted for the apoenzymes indicates moderate conservation of primary structure. Overall the enzymes display 15% sequence identity; however, isolated regions display significantly greater sequence homology, most notably the carboxyl-terminal 150 amino acids where 30% of residues are identical.

All photolyases share at least three common functions: (i) binding of the ubiquitous FADH₂ chromophore; (ii) binding of a second chromophore, which, depending upon the source of the enzyme, is either 5,10-methenyltetrahydrofolate (E. coli, S. typhimurium, N. crassa, S. cerevisiae) or a derivative of 8-hydroxy-5-deazafavin (A. nidulans, H. halobium, S. griseus); (iii) specific binding to pyrimidine dimers. Surprisingly, neither flavin, folate, nor DNA binding domains are apparent from the primary sequences of the enzymes. Recent studies by Malhotra et al. (1992) on the Phr1 photolyase of S. cerevisiae demonstrated that the carboxyl-terminal 275 residues of the enzyme comprise the FAD binding domain, whereas residues 15-326 contain the folate binding domain. However neither of the fragments comprising the chromophore binding domains was capable of binding dimer-containing DNA with high affinity and specificity. We report here the results of chemical modification and site-directed mutagenesis studies which have identified residues involved in substrate binding and discrimination by Phr1. Our results implicate 4 conserved amino acids (Lys617, Arg69, Lys642, and Trp387) in substrate binding, thereby establishing that at least a portion of the DNA binding site is composed of residues in the conserved carboxyl-terminal domain. Alanine substitution at each of these sites reduces the affinity of photolyase for substrate and produces specific changes in the interactions between Phr1 and DNA phosphates and bases surrounding the dimer. The implications of these results for models of substrate recognition and discrimination by photolyases are discussed.

EXPERIMENTAL PROCEDURES

Materials—Sources of materials were the same as reported in our previous study (Baer and Sancar, 1989). Micrococcus luteus pyrimidine dimer glycosylase-AP endonuclease was purchased from Applied Genetics Inc., oligo(dT)₃₀ was from Pharmacia LKB Biotechnology Inc., and hydroxylapatite Ultrogel was from IBF Biotechnics. Acrylamide for quenching experiments was obtained from American BioNuclear and was washed twice in ice-cold 95% ethanol prior to use.

Enzyme Preparations—All photolyases were expressed from tac-regulated cloned genes carried on derivatives of plasmid pCB1241 (Sancar and Smith, 1988), propagated in E. coli strain CSR603/ F′lac⁺ (Sancar, 1985a). The enzymes were purified as described previously (Sancar et al., 1987a) with the following modifications. After elution from DNA cellulose, fractions containing photolyase were pooled, dialyzed into hydroxyapatite column buffer (20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol) and loaded onto a hydroxyapatite column (16 × 48 mm) equilibrated in the same buffer. Following a 50-ml wash with column buffer, a 50-ml linear gradient of 20-400 mM potassium phosphate was applied. One-ml fractions were collected and 10 μl from each fraction were analyzed by SDS-polyacrylamide

* This work was supported by Grant GM35123 from the National Institute of General Medical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ To whom correspondence should be addressed.

This is an Open Access article under the CC BY license.
gel electrophoresis (Laemmli, 1970). Photolyase began eluting at 35% of the gradient. Fractions containing the enzyme were pooled and the enzyme was concentrated using Centriprep 30 (Amicon) concentrators, then dialyzed either into photolyase storage buffer (Sancar et al., 1987a) or into a sucrose buffer (Jorns et al., 1984) for spectroscopic and chromatographic characterization. Photolyase chromatography was monitored by spectroscopy as described previously (Sancar et al., 1987a) using the following extinction coefficients: εmax = 2.8 × 104 M⁻¹ cm⁻¹ for holoenzyme (Johnson et al., 1988), εmax = 11.3 × 104 M⁻¹ cm⁻¹ for FAD₄₄ and εmax = 12.5 × 104 M⁻¹ cm⁻¹ for apoenzyme (Sancar et al., 1987a).

**Photolyase Binding Reaction**—All binding reactions were performed at 23 °C in a 25-μl mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM β-mercaptoethanol, 6% (v/v) glycerol, and 5 × 10⁻⁶ M substrate DNA. The substrate was a 43-bp oligonucleotide (shown below), with a centrally located thymine dimer (indicated by <-) and was constructed as described previously (Husain et al., 1987; Baer and Sancar, 1988) with 32P label at the 5' end of the top strand. After a 20-min incubation at room temperature, free DNA and enzyme-bound DNA were separated on the basis of electrophoretic mobility (EMSA; Fried and Crothers, 1981; Garner and Revzin, 1981). Reaction mixtures were dialyzed either into photolyase storage buffer (Sancar et al., 1989) with 32P label at the 5' end of the top strand. After a 15-min incubation at 15 °C, free enzyme was removed by centrifugation through an LKB RackBeta instrument employing a predetermined correction for spectral overlap. Isolated peptides were sequenced by Dr. David Clapper (University of North Carolina at Chapel Hill) using an Applied Biosystems automated peptide sequencer with an in-line Waters HPLC. Each amino acid peak was collected and analyzed for 14C and 3H content.

**Plasmid Constructions**—Phage M13 mp18:PHR1Kpn, which contains bp 1363–2250 of PHR1 (Sancar, 1985b), was constructed as follows. A 2.4-kbp PvuI-PstI fragment containing PHR1 was excised from pCB105 (Sancar and Smith, 1988) and ligated into Smal-PstI-digested M13 mp18 to yield M13 mp18:PHR1Kpn. Single-stranded template was made in the E. coli strain CJ236 (6ut-1, ung-1, thi-1, relA-1, pCJ105(Cm)), and oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to remove a BglII site at position 1710 (Sancar, 1985b) and a unique BglII site at position 959 (Baer et al., 1987a) except that photolyase concentration was determined by scintillation counting in an LKB RackBeta instrument employing a predetermined correction for spectral overlap. Isolated peptides were sequenced by Dr. David Clapper (University of North Carolina at Chapel Hill) using an Applied Biosystems automated peptide sequencer with an in-line Waters HPLC. Each amino acid peak was collected and analyzed for 14C and 3H content.

**Preparative Scale Reductive Methylation of Phr1—Oligo(DT)₃₀** containing five dimers/molecule was prepared by irradiating a solution of oligonucleotide (2 × 10⁻⁶ M in 10 mM NaCl) with 254-nm light. Total dimer content was determined by the decrease in absorbance at 280 nm. Three nmol of Phr1 in 100 μl were incubated with 100 mM [H]HCHO (80 μCi/mmol) as described previously by Takeda et al. (1986). In a similar reaction 100 mM [¹⁴C]HCHO (10 mCi/mmol) was used to modify Phr1 in the presence of 1 × 10⁻⁶ M dimer-containing oligo(DT)₃₀ (dimer) × 5 × 10⁻⁴. Greater than 90% of enzyme was bound to substrate under these conditions. One minute after addition of labeled HCHO, 5 volumes of nonlabeled 100 mM formaldehyde were added to both reactions. In a third reaction 900 nmol of Phr1 in 300 μl were reductively methylated using 100 mM unlabeled formaldehyde. Ten min after addition of unlabeled formaldehyde, all reactions were mixed with 100 mM unlabeled formaldehyde, the mixtures were combined, and photolyase was precipitated at room temperature by addition of trifluoroacetic acid to a final concentration of 1% (v/v). The precipitate was recovered by centrifugation at 16,000 × g for 10 min, dissolved in 10 mM guanidine hydrochloride, and dialyzed into 10 mM NaCl at 4 °C. During dialysis, Phr1 precipitated. Enzyme yield was determined by centrifugation as described above, dissolved in 200 μl of 70% formic acid (v/v), and treated with 200 mM CNBr under argon for 24 h at room temperature. The digest was lyophilized and dissolved in 250 μl of 0.1% trifluoroacetic acid (v/v).

Peptides were separated by HPLC using a W-Forex (Phenomenex) reverse phase C18 column (250 × 4.6 mm). Samples were loaded onto a column pre-equilibrated with 0.1% (v/v) trifluoroacetic acid, then peptides were eluted with a 60-mL linear gradient of 0–70% (v/v) methanol. Peaks were collected manually. Multiple runs at various flow rates were used to separate closely eluting peptides which were detected by absorbance at 220 nm using an in-line ultraviolet detector.

**Kinetics of Inactivation of Phr1 by Reductive Methylation**—The method used for reductive methylation of lysines in Phr1 was based on that of Cabacungan et al. (1982). Photolyase (2.4 × 10⁻⁷ M) was incubated at room temperature in 250 μl of 100 mM formaldehyde in modification buffer (50 mM Hepes buffer, pH 7.4, 100 mM KCl, 6% (v/v) glycerol, and 20 mM NaCNBH₃). At various times 20-μL samples were removed and 2.5 μl of 1.0 M lysine were added to quench the reaction. Labeled substrate (2.5 μl of 10⁻⁷ M) was then added to the samples and incubated for 10 min prior to EMSA. In a similar experiment, photolyase was incubated with labeled substrate (modified) for 10 min at room temperature prior to formylation of lysine, and 20-μL aliquots were removed at various times. Five μl of 500 mM lysine were added to quench the reaction. Bound and free substrate were separated by EMSA and quantitated.

**Preparative Scale Reductive Methylation of Phr1—Oligo(DT)₃₀** containing five dimers/molecule was prepared by irradiating a solution of oligonucleotide (2 × 10⁻⁶ M in 10 mM NaCl) with 254-nm light. Total dimer content was determined by the decrease in absorbance at 280 nm. Three nmol of Phr1 in 100 μl were incubated with 100 mM [H]HCHO (80 μCi/mmol) as described previously by Takeda et al. (1986). In a similar reaction 100 mM [¹⁴C]HCHO (10 mCi/mmol) was used to modify Phr1 in the presence of 1 × 10⁻⁶ M dimer-containing oligo(DT)₃₀ (dimer) × 5 × 10⁻⁴. Greater than 90% of enzyme was bound to substrate under these conditions. One minute after addition of labeled HCHO, 5 volumes of nonlabeled 100 mM formaldehyde were added to both reactions. In a third reaction 900 nmol of Phr1 in 300 μl were reductively methylated using 100 mM unlabeled formaldehyde. Ten min after addition of unlabeled formaldehyde, all reactions were mixed with 100 mM unlabeled formaldehyde, the mixtures were combined, and photolyase was precipitated at room temperature by addition of trifluoroacetic acid to a final concentration of 1% (v/v). The precipitate was recovered by centrifugation at 16,000 × g for 10 min, dissolved in 6 M guanidine hydrochloride, and dialyzed into 10 mM NaCl at 4 °C. During dialysis, Phr1 precipitated. Enzyme yield was determined by centrifugation as described above, dissolved in 200 μl of 70% formic acid (v/v), and treated with 200 mM CNBr under argon for 24 h at room temperature. The digest was lyophilized and dissolved in 250 μl of 0.1% trifluoroacetic acid (v/v).

Peptides were separated by HPLC using a W-Forex (Phenomenex) reverse phase C18 column (250 × 4.6 mm). Samples were loaded onto a column pre-equilibrated with 0.1% (v/v) trifluoroacetic acid, then peptides were eluted with a 60-mL linear gradient of 0–70% (v/v) methanol. Peaks were collected manually. Multiple runs at various flow rates were used to separate closely eluting peptides which were detected by absorbance at 220 nm using an in-line ultraviolet detector.

1 The abbreviations used are: bp, base pair(s); kb, kilobase pair; EMSA, electrophoretic mobility shift assay; DMS, dimethyl sulfate; HPLC, high performance liquid chromatography.
then challenged with 32P-labeled dimer-containing 43-bp substrate, and bound and free substrate were separated by EMSA. Remaining binding activity was assessed by EMSA using 32P-labeled 43-bp substrate; V, Phrl treated in the presence or absence of substrate and samples were removed at the indicated times. Remaining binding activity was assessed by EMSA using 32P-labeled 43-bp substrate; V, Phrl treated in the presence of nondimer DNA; O, Phrl(Lys617 → Ala) treated in the presence of dimer-containing DNA; ●, Phrl treated in the absence of substrate; ●, Phrl treated in the presence of nondimer DNA; O, Phrl(Lys617 → Ala) treated in the presence of dimer-containing substrate; ■, Phrl(Lys617 → Ala) treated in the absence of substrate. Phrl was at 9.4 × 10⁻⁸ M and Phrl(Lys617 → Ala) at 2.4 × 10⁻⁸ M.

lyase was incubated with substrate such that 80% of the substrate was bound. The reaction mixture was exposed to successive 500 erg/mm² fluences of 380 nm radiation, 20-µl aliquots were removed after each irradiation and photolyase-bound substrate and free substrate were separated by EMSA and quantitated. Quantum yields were determined by plotting total fluorescence received versus the fraction of ES complexes remaining (Harm and Rupert, 1968). An alternative method for determining repair was used in some cases. 10-µl aliquots were removed after each irradiation, extracted with phenol, dissolved in buffer (50 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA), and treated for 90 min at 37 °C with 320 units of dimer glycosylase-AP endonuclease, which cleaves the intradimer phosphodiester bond. Control experiments indicated that all nonrepaired substrate was cleaved under these conditions. Cleaved and full-length (repaired) substrates were separated on a 12% sequencing gel and quantitated.

Acrylamide Quenching—Fluorescence quenching experiments were performed using a Shimadzu RF5000U spectrophotometer at 15 °C as described by Kim et al. (1992). 1.5 × 10⁻⁸ M photolyase in 100 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5, was exposed to 256 nm radiation, and tryptophan fluorescence emission was monitored at 334 nm. Aliquots of 7 M acrylamide were added to the solution and after each addition fluorescence at 334 nm recorded. The data were analyzed using the modified Stern-Volmer equation (Lakowicz, 1983), F₀/F₀ = 1/f + fKₜ/SQ, where F₀ and F are, respectively, the relative fluorescence intensities without and with quencher at concentration [Q], f is the fractional accessibility of the tryptophans in photolyase to quenching agent, and Kₜ/SQ is the Stern-Volmer constant which reflects the efficiency of quenching. Kₜ/SQ was determined from the slope of a plot of (F₀ – F)/F₀ versus 1/Q.

RESULTS

Reductive Methylation of Phrl—Phrl interacts extensively with DNA phosphates on the dimer-containing strand (Baer and Sancar, 1989) and displaces 3-4 Na⁺ upon binding. Because interactions between proteins and DNA phosphates frequently involve salt bridge formation (Siebenlist and Gilber, 1980), we decided to examine the effect of lysine modification on substrate binding. Treatment of proteins with formaldehyde in the presence of sodium cyanoborohydride results in reductive methylation of solvent exposed lysines (Cabanacung et al., 1982). Phrl was treated for various times, then challenged with 32P-labeled dimer-containing 43-bp substrate, and bound and free substrate were separated by EMSA. As can be seen in Fig. 1, DNA binding activity was rapidly lost; the initial slope yielded a t₁/₂ of 1.8 min. In contrast, when Phrl was incubated with substrate such that 80% of substrate and enzyme were bound, then treated with formaldehyde, t₁/₂ increased to 5.0 min. Protection was not observed when Phrl was treated in the presence of an identical concentration of 43-bp substrate lacking pyrimidine dimer. Thus substrate binding protects one or more lysines in Phrl from methylation.

To identify the specific lysines protected, Phrl was treated with [3H]formaldehyde in the absence of DNA and [14C]formaldehyde in the presence of dimer-containing oligo-(dT)₁₈. The enzymes were then mixed, cleaved with CNBr, and the resulting peptides were separated by HPLC. In the initial separation 57 peaks were detected (Fig. 2, panel A); this is approximately three times the number of expected peaks (Phrl contains 18 methionines) and was due to incomplete cleavage by CNBr. Both the intact protein (data not shown), and the majority of peaks displayed approximately a 1:1 ratio of 3H:14C (Fig. 2, panel B). To ensure that peaks with elevated 3H:14C ratios were not missed due to peak overlap, all peaks with ratios >0.9 were repurified. Upon repurification peak 38 displayed a 3H:14C ratio of 2.0 (Fig. 2, panels C and D), indicating the presence of a lysine protected from modification in the ES complex; all other peaks displayed ratios <1.3 and were not examined further. Sequence analysis of the peak 38 peptide and scintillation counting of the cleaved phenylthiohydantoin derivatives revealed that the protected lysine was at position 517 in the Phrl sequence (Fig. 3).

To confirm that Lys517 plays a role in DNA binding, this residue was changed to an alanine by site-directed mutagenesis, and the mutant enzyme was purified to apparent homogeneity. As can be seen in Fig. 1, substrate DNA did not protect Phrl(Lys517 → Ala) from inactivation by reductive methylation. The t₁/₂ for inactivation in both the presence and absence of substrate was 1.0 min. Therefore, we conclude that methylation of Lys517 in the wild type enzyme is responsible for the initial loss of binding activity. In all sequenced microbial photolyses the residue at the equivalent position is either Lys or Arg (Fig. 3), suggesting a strict functional requirement for a positive charge at this position.

Construction of Phrl Mutants and Equilibrium Binding Analysis—Lys517 lies in the conserved carboxyl-terminal region of Phrl, suggesting that this region comprises at least a portion of the DNA binding domain of the enzyme. We therefore introduced mutations at additional conserved residues within this region and determined the effect on specific and nonspecific binding to substrate. Arg520 is conserved in all reported microbial photolyase sequences and is located near Lys517, whereas Lys517 is the only lysine that is conserved in all microbial photolyase sequences. Trp387 was also targeted because previous work on E. coli photolyase (Li and Sancar, 1990) has implicated the equivalent Trp residue in DNA binding. In all cases the original amino acid was replaced with an alanine residue, which is equally accommodated in hydrophobic and hydrophilic environments (Schultz and Schrimer, 1979). Each mutant photolyase was overproduced at near wild type levels and purified to apparent homogeneity. Wild type Phrl (Sancar et al., 1987a) and the mutant photolyses displayed similar absorbance spectra with peaks at 277 and 377 nm (data not shown). In addition each mutant enzyme contained folate, flavin, and apoenzyme in 1:1:1 stoichiometry, and in no case were there detectable quantities of flavin radical or FAΔ₈ (data not shown). We conclude that the amino acid substitutions do not introduce large structural perturbations in the enzyme which might be expected to

G. B. Sancar and F. W. Smith, manuscript in preparation.
destabilize chromophore binding or alter the oxidation states of the chromophores.

Specific (K<sub>s</sub>) and nonspecific (K<sub>ns</sub>) equilibrium binding constants were determined for each mutant. As can be seen in Table I, each substitution had a pronounced effect on both parameters. Compared with wild type PhrI, K<sub>s</sub> for PhrI(Lys<sup>517</sup> → Ala) decreased by an order of magnitude, whereas K<sub>s</sub> for PhrI(Arg<sup>407</sup> → Ala), PhrI(Lys<sup>645</sup> → Ala) and PhrI(Trp<sup>387</sup> → Ala) decreased by 2 orders of magnitude. Thus each of the mutant photolyases has reduced affinity for dimer-containing DNA. The discrimination ratio (K<sub>s</sub>/K<sub>ns</sub>) is a measure of the ability of a DNA binding protein to selectively bind to its target. The discrimination ratio for wild type PhrI is 10<sup>4</sup>. PhrI(Lys<sup>517</sup> → Ala) displayed a similar discrimination ratio while PhrI(Arg<sup>407</sup> → Ala), PhrI(Lys<sup>645</sup> → Ala), and PhrI(Trp<sup>387</sup> → Ala) had discrimination ratios of approximately 10<sup>3</sup>. This suggests that interactions involving Lys<sup>517</sup> contribute equally to specific and nonspecific binding, whereas Arg<sup>407</sup>, Lys<sup>645</sup>, and Trp<sup>387</sup> participate in interactions which contribute to a greater extent to substrate discrimination.

**Phosphate Contacts of Mutant Photolyases—Ethynitrosourea ethylates phosphate oxygens on the DNA backbone and interferes with photolyase binding to dimer-containing DNA either by eliminating ionic interactions between PhrI and DNA or by steric hindrance (Baer and Sancar, 1989). To map the sites of altered interactions between the mutant photolyases and DNA phosphates, we compared the ethylation interference patterns of the mutant and wild type enzymes. The results are shown in Fig. 4. As in our previous work (Baer and Sancar, 1989) we found that, for wild type PhrI, ethylation of the phosphate immediately 5' to the dimer weakly inhibited binding, whereas ethylation of any of the three phosphates immediately 3' to the dimer strongly inhibited binding. Ethylation at the fourth phosphate 3' to the dimer also inhibited binding by PhrI but to a lesser extent. Interaction at the 5' site was substantially weaker than in our previous study; this probably reflects the fact that the current experiments were performed with saturating enzyme concentrations (see “Experimental Procedures”). The interference pattern obtained with PhrI(Lys<sup>517</sup> → Ala) was identical

![Graphs](image_url)

**FIG. 2.** HPLC profiles and <sup>3</sup>H:<sup>14</sup>C ratios for CNBr-cleaved photolyase and for the repurified peak 38 material. PhrI was treated with [<sup>3</sup>H] and [<sup>14</sup>C]formaldehyde in the absence or presence of substrate, respectively, and the treated samples were combined. Following removal of unincorporated counts and treatment with CNBr, peptides were applied in 0.1% (v/v) trifluoroacetic acid to a reverse phase C18 column and were eluted with a linear gradient of 0–70% (v/v) methanol in 0.1% (v/v) trifluoroacetic acid. Panels A and B show, respectively, the absorbance at 220 nm and <sup>3</sup>H:<sup>14</sup>C ratios obtained during the initial fractionation of CNBr-treated PhrI. The first 17 fractions were collected across the breakthrough peak and appeared to contain primarily large partial digestion products (data not shown). Panels C and D show the absorbance and <sup>3</sup>H:<sup>14</sup>C ratios obtained upon repurification of material from peak 38 of the initial separation. The arrows in panels A and C indicate the locations of peak 38. Purified material from the separation shown in panel C was subjected to amino acid sequence analysis.

**FIG. 3.** Sequence of the COOH-terminal third of PhrI (adapted from Sancar, 1985b). Underlining indicates the isolated peptide with a <sup>3</sup>H:<sup>14</sup>C ratio of 2:1 in the reductive methylation experiments. The lysine protected from reductive methylation at position 517 is indicated by an asterisk. Amino acids that were mutated to alanine are indicated by a dot. Residues identical in five out of seven microbial photolyases are shown in uppercase letters.

**TABLE I**

| Enzyme  | K<sub>s</sub> | K<sub>ns</sub> | ΔΔG<sub>observed</sub> | ΔΔG<sub>calculated</sub> |
|---------|-------------|---------------|----------------------|------------------------|
| Wild type | 3.7 × 10<sup>-4</sup> | 2.6 × 10<sup>-4</sup> | -12.8 | 0 |
| PhrI(Lys<sup>407</sup> → Ala) | 3.3 × 10<sup>-4</sup> | 2.3 × 10<sup>-4</sup> | -11.4 | +1.4 |
| PhrI(Trp<sup>407</sup> → Ala) | 4.1 × 10<sup>-4</sup> | 1.1 × 10<sup>-4</sup> | -10.2 | +2.6 |
| PhrI(Trp<sup>387</sup> → Ala) | 1.7 × 10<sup>-4</sup> | 8.0 × 10<sup>-4</sup> | -9.7 | +3.1 |
| PhrI(Lys<sup>387</sup> → Ala) | 4.0 × 10<sup>-4</sup> | 1.4 × 10<sup>-4</sup> | -10.2 | +2.6 |
Additionally, methylation interference at G27 was also absent for this mutant. Because interference at the latter site is normally weak for wild type Phr1, we believe that the primary site at which interaction is perturbed is at G21 and the adjacent phosphate (see above). As was evident in the ethylation interference studies, multiple interactions are affected in the Phr1(Trp587→Ala) and Phr1(Lys463→Ala) complexes. Methylation at G21 did not interfere with binding by either mutant, whereas, in contrast to wild type, methylation of G24 interfered with binding by Phr1(Trp587→Ala). Thus interactions with the bases immediately flanking the dimer are altered in the Phr1(Trp587→Ala) ES complex. Substrate binding by Phr1(Lys463→Ala) appeared to be insensitive to methylation as indicated by the absence of interference at any site. Because photolyase binds pyrimidine dimers regardless of the surrounding sequence context (Myles et al., 1987; Rupert, 1962a, 1962b), methylation interference probably reflects steric constraints rather than the loss of hydrogen bonds between the enzyme and DNA bases. The results indicate that interactions with major groove residues are altered in each mutant Phr1·ES complex.

Relative Quantum Yield—The quantum yield for photolysis of dimers by Phr1 at 384 nm is 0.5 and proceeds via energy transfer from the folate chromophore to FADH2 followed by electron transfer from flavin to the dimer (Sancar, 1990). This process is potentially sensitive to changes in the alignment of and distance between enzyme-bound FADH2 and the dimer. Additionally, changes in enzyme structure which alter interaction between the folate chromophore and FADH2 may affect the quantum yield of photolysis. To further characterize the substitution mutants, we determined the quantum yield for each mutant enzyme relative to wild type. As can be seen in Table II, the quantum yields displayed by two of the mutants, Phr1(Trp587→Ala) and Phr1(Lys463→Ala), were substantially different from wild type. To confirm these differences, we used a more direct method for quantitating repair. After the reaction mixtures were exposed to photoreactivating light, each sample was treated with M. luteus pyrimidine dimer glycosylase-AP endonuclease which cleaves the glycosyl bond linking the 5' dimer base and sugar as well as the intradimer phosphodiester bond (Haseltine et al., 1980). Full-length and cleaved substrate were separated on a 12% sequencing gel. When the quantum yield was calculated by this method, the value obtained for Phr1(Trp587→Ala) was one-third that of wild type Phr1. This is in contrast to results reported by Li and Sancar (1990) which indicate that (different) mutations at the equivalent site in E. coli photolyase do not affect the quantum yield. In contrast the quantum yield displayed by Phr1(Lys463→Ala) was similar to wild type. We believe that the latter value for this mutant is the correct one and that the greater value obtained in the EMSA analysis reflects denaturation of the enzyme during irradiation and/or electrophoresis; the released substrate would be scored as repaired in the EMSA assay. This is consistent with the fact that preparations of this enzyme consistently displayed the lowest number of active molecules in the binding assay (40%).

In addition the known reaction mechanism for photolysis precludes a quantum yield greater than 1.0, which is implied by the results of the EMSA experiment.

Conformational Analysis of Phr1 and Photolyase Mutants by Acrylamide Quenching—The intrinsic fluorescence of solvent-exposed tryptophan residues is quenched by acrylamide. Thus structural perturbations which expose one or more tryptophans to solvent can be detected by both increased tryptophan fluorescence and increased quenching by acrylamide. To test whether any of the mutations introduced into
Substrate Binding by Photolyase

FIG. 5. Methylation interference analysis of wild type and mutant photolyases. Substrate was treated with DMS, mixed with photolyase, and bound and free substrate were separated by EMSA and analyzed on a 12% DNA sequencing gel. Panel A shows a typical autoradiograph of the results obtained. Dash indicates substrate treated with DMS with no photolyase present. Labeling of the lanes and bands is the same as in Fig. 4. Filled circles between lanes indicate sites of methylation interference with wild type enzyme and which are unchanged in the mutants. Open circles indicate positions at which interference is lost or gained in the mutants. The asterisk indicates the band used to correct for variations in loading. Panel B shows the relative band intensity for the affected residues. Positive values indicate methylation interference and negative values indicate binding stimulation. Only absolute values $>0.1$ were considered significant.

**TABLE II**
Photochemical characteristics of wild type and mutant photolyases

| Enzyme           | Relative Quantum Yield at 380 nm | $K_{sv}$ $^a$ |
|------------------|----------------------------------|---------------|
|                  | EMSA $^a$                      | M. luteus $^a$ |             |
| Wild type        | 1.0                             | 1.0           | 3.5          |
| Phr1(Lys857→Ala) | 1.2                             | ND $^d$       | 3.0          |
| Phr1(Arg897→Ala) | 0.8                             | ND $^d$       | 3.5          |
| Phr1(Lys930→Ala) | 2.0                             | 0.9           | 2.9          |
| Phr1(Try987→Ala) | 0.3                             | 0.3           | 3.1          |
| Tryptophan*      |                                  | 20.0          |             |

* Fluorescence emission measured at 334 nm for native enzyme and at 352 nm for tryptophan.
$^d$ EMSA, repair determined by electrophoretic mobility shift assay.
$^d$ Repair determined by endonuclease-sensitive sites using M. luteus pyrimidine dimer glycosylase-AP endonuclease.
$^d$ ND, not done.

Concentration $= 2.7 \times 10^{-5}$ M, which was the same as the tryptophan concentration in the photolyase samples.

Phr1 had greatly perturbed the structure of the enzyme, acrylamide quenching experiments were performed. This method is particularly suited for analysis of the mutants described here as the carboxyl-terminal region of Phr1, in which the mutations are located, contains 9 tryptophan residues distributed throughout the region. The enzymes were exposed to 295 nm radiation, and fluorescence emission at 334 nm was measured. Addition of small aliquots of 7 M acrylamide quenched the fluorescent emission of the tryptophans and permitted us to determine $K_{sv}$, which reflects the efficiency of quenching. $K_{sv}$ for all mutant photolyases was similar to wild type Phr1, and quenching was insignificant compared with that seen with an equimolar concentration of free tryptophan (Table II).

**DISCUSSION**
DNA photolyases repair pyrimidine dimers in a variety of sequence contexts yet discriminate as efficiently between target and nontarget sequences as do sequence-specific DNA binding proteins (Rupert, 1962a, 1962b; Sancar et al., 1987b; Sancar, 1990). Unlike the latter proteins, which utilize primarily sequence-specific base contacts to identify target sequences, photolyases must recognize an altered DNA structure introduced by and characteristic of a pyrimidine dimer. This structure includes the two pyrimidines in the dimer, as shown by the observation that dimer-containing UpU, TpT,
and the TT base dimer are repaired by *E. coli* photolyase (Wittmer et al., 1989; Kim and Sancar, 1991). However additional interactions with flanking residues are at least as important in stabilizing the ES complex; comparison of the $K_a$ for binding to dimer-containing poly(dT) versusTpT indicates that 50% of the binding free energy comes from such flanking interactions (Kim and Sancar, 1991). More importantly, the specific binding equilibrium constant ($K_a$) decreases with substrate length, indicating that at least some of these flanking interactions are utilized in substrate discrimination. Mapping of the contacts made by Phr1 on the DNA surrounding the dimer has established that residues on yeast photolyase lie in close proximity to or interact with four to five phosphates on the dimer-containing strand as well as with major groove residues 5' and 3' to the dimer (Baer and Sancar, 1989). The same contacts are utilized by photolyases from a prokaryote (*E. coli*; Husain et al., 1987) and an archaebacterium (*Methanobacterium thermoautotrophicum*; Kiefer et al., 1989), suggesting that all photolyases recognize the same structural determinants which uniquely specify the site of a dimer. However previous studies have not revealed which interactions play a role in binding specificity nor have the amino acids involved in specific substrate recognition by Phr1 been identified. In the present study we have addressed these questions by constructing and characterizing a set of mutants which are defective in substrate binding and discrimination and which have lost specific contacts to residues on the DNA surrounding the dimer.

Alanine substitution mutagenesis was utilized in this study in an attempt to minimize the potential for structural perturbation of the enzyme. Consistent with this, several lines of evidence indicate that the Phr1 mutants described here have not suffered extensive structural alterations. (i) Each protein contains a complete complement of the flavin and folate chromophores, and (ii) the redox states of the chromophores are unchanged. Thus the chromophore binding sites as well as the interactions which maintain the chromophores in the reduced state are not altered. (iii) With the exception of Phr1 (Trp377 → Ala) the quantum yield for photolysis for each mutant is similar to that of wild type photolyase, indicating that the interactions between the two chromophores and between the flavin chromophore and the pyrimidines in the dimer are not significantly changed. (iv) With the exception of Phr1 (Lys517 → Ala), the fraction of photolyase molecules active in DNA binding exceeded 70% in each preparation (data not shown). (v) All mutants are able to discriminate to a significant extent between dimer and non-dimer DNA. (vi) All mutants display a response to acrylamide quenching indistinguishable from wild type, suggesting that at most only minor local structural changes have resulted from the substitutions.

Each of the mutations reported here produces a characteristic decrease in the free energy of binding, with Δ$G_{bio}$ ranging from +1.4 to +3.1 kcal/mol (Table 1). Fersht (1989) has estimated that a single hydrogen bond between uncharged groups contributes 0.5-1.5 kcal/mol to binding energy, whereas a hydrogen bond to a charged group contributes 3-4 kcal/mol. Similarly in 0.1 M NaCl a single electrostatic interaction between a DNA phosphate and the ϵ-amino group of lysine contributes 1.2 kcal/mol (Record et al., 1976). Thus the changes in binding energy that we observe are consistent with loss of only one or a few contacts. In addition the mutants displaying the greatest losses in binding energy display the greatest number of altered interactions in methylation and ethylation interference assays. This is strong evidence that the changes in $K_a$ reflect primarily altered interactions at the DNA-photolyase interface. The sites of altered interactions that we have detected are summarized in Fig. 6.

Lys517 is protected from methylation when Phr1 is bound to substrate but not when the enzyme is free in solution, implying that it is buried between Phr1 and DNA in the ES complex. Furthermore the fact that, in contrast to wild type Phr1, the initial kinetics of inactivation of Phr1(Lys517 → Ala) are unaffected by substrate binding indicates that the rapid loss of binding activity for wild type Phr1 is the consequence of methylation of Lys517. Further evidence for the involvement of Lys517 in binding comes from thermodynamic and binding interference analysis. Both the specific and non-specific equilibrium binding constants are reduced 10-fold for Phr1(Lys517 → Ala); thus interactions involving Lys517 contribute approximately equally to the free energy of specific and nonspecific binding. Loss of methylation interference at G25 suggests that Lys517 interacts with the second base 3' to the dimer. It is unlikely that the decrease in binding energy exhibited by Phr1(Lys517 → Ala) reflects loss of a hydrogen bond between N7 of G25 and Phr1; the lack of sequence specificity of photolyase binding implies the absence of sequence-specific hydrogen bonds with the bases surrounding the dimer. Rather the methyl groups of the Lys517 side chain may participate in van der Waals interactions with G25 and methylation at N7 may sterically interfere with binding of Phr1. This does not rule out additional interactions between the ϵ-amino group of Lys517 and DNA at sites not currently amenable to interference analysis, for example the intradimer phosphate, adjacent ribose moieties, or O' of the thymines in the dimer.

Phr1(Arg307 → Ala) is the only other mutant in this study that exhibits a small number of changes in the interference pattern. Ethylation interference at the first phosphate 5' to the dimer is absent from this mutant, as is methylation interference at G21, the base immediately 5' to the dimer. Loss of these interactions is accompanied by a 100-fold decrease in $K_a$ and a 20-fold decrease in $K_{SR}$, indicating that Arg307 contributes to substrate discrimination. Although interactions between positively charged amino acid side chains and DNA phosphates have usually been considered "nonspecific," studies on substrate binding by EcoRI endonuclease have demonstrated that such interactions can serve to "anchor and orient protein recognition elements" and thus make a significant energetic contribution to substrate discrimination (Lesser et al., 1990). Our results suggest that interaction between Arg307 and the phosphate 5' to the dimer plays such a role in photolyase binding. Discrimination probably entails recognition of the position of phosphoryl oxygens, which is influenced by local DNA conformation. This is consistent with the currently accepted model of dimer-containing DNA in which the conformation of the sugar-phosphate backbone is altered from the first phosphate 5' to the dimer to at least

**Fig. 6. Summary of major groove and phosphate contacts made by wild type and mutant photolyases.** Guanine (major groove) and phosphate contacts are indicated by white letters in filled boxes.
demonstrated that the tripeptide Lys-Trp-Lys can bind to the binding site in the absence of DNA with high quantum yield, implying that TrpZ7' of Phr1 plays a crucial role in DNA binding for Phr1.

Replacement of either Trp377 or Lys463 disrupts multiple DNA contacts and many of the affected sites are identical in the two mutants. For both, interference is lost at the first and phosphate 5' to the dimer as well as at the two phosphates immediately 3' to the dimer. In addition methylation of the second and fourth G's 3' to the dimer (G25 and G27) fails to inhibit binding of Phr1(Lys463 → Ala). The large number of changes resulting from these single amino acid substitutions suggests that Trp377 and Lys463 play a crucial role (direct or indirect) in orienting many of the Phr1 specific determinants at the binding site. In the case of Lys463, current evidence does not permit us to discriminate between a role in maintaining the local secondary structure of the binding site in the absence of DNA versus participation in a network of interdependent interactions between Phr1 and DNA. However studies on E. coli photolyase suggest a direct role in DNA binding for Trp377. Based on alignment of the amino acid sequences, Trp377 in the bacterial enzyme is the homologue of Trp377 of Phr1. Kim et al. (1992) have shown that Trp377 of E. coli photolyase photocatalyzes photoreactivation at 280 nm with high quantum yield, implying that Trp377 lies in close proximity to the DNA. In addition a Trp377 → Arg mutation decreases Km 200-fold and increases Kcat 5-fold (Li and Sancar, 1990). Helene and Maurizot (1981) have demonstrated that the tripeptide Lys-Trp-Lys can bind to DNA via nonspecific interactions between the 2 Lys residues and DNA phosphates and intercalation of the central Trp residue. Because intercalation is only partial, the bases flanking the insertion site are forced open at an angle and the helix is kinked. This is similar to the altered structure predicted at the two pyrimidines in the dimer (Pearlman et al., 1985) and suggests that intercalation of Trp at the dimer may be energetically favored and thus contribute to substrate discrimination. A number of altered contacts surrounding the dimer in the Phr1(Trp377 → Ala) ES complex may reflect the importance of intercalation in correctly orienting additional recognition elements.

Phr1(Trp377 → Ala) also displays a 3-fold reduction in the quantum yield for photolyis at 380 nm. Given the disruption of normal interactions at the DNA-protein interface, we believe that this is the indirect result of misalignment of the enzyme-bound FADH2 and the dimer in an abnormal "adaptive" ES complex. In contrast, Li and Sancar (1990) have reported that substitution of Trp277 of E. coli photolyase with Arg, Glu, Phe, or His does not significantly affect the quantum yield, despite the fact that both the Trp277 → Arg and Trp277 → Glu mutants are clearly defective in substrate binding and discrimination. At present the reason for the different effects of mutations in the two enzymes is not clear.

Each of the four sites probed by site-directed mutagenesis in this study is conserved in all photolyases characterized to date, with the exception of photolyase from the goldfish Carassius auratus (Yasuhira and Yasui, 1992). (The predicted amino acid sequence for C. auratus photolyase is unlike that of the microbial enzymes.) Thus it is likely that these residues are part of a dimer recognition motif which is common to most or all DNA photolyases. Solution of a photolyase-DNA cocrystal structure will be necessary to establish whether these residues directly contact the DNA or anchor other side chains which make these contacts. In either case, the results reported here establish functional roles for Trp377, Lys463, Arg377, and Lys417 in substrate binding and discrimination and suggest some of the sites on dimer-containing DNA that are involved in these processes. Any proposed photolyase structure must be consistent with these observations.

Acknowledgments—We thank Ariz Sancar and Sang-Se-Tae Kim for insightful discussions.

REFERENCES

Baer, M., and Sancar, G. B. (1988) Mol. Cell. Biol. 9, 4777-4786
Cabardou, J., Chambon, S., and Ansel, M. L. (1985) Anal. Biochem. 142, 272-278
Feder, A. R. (1988) Trends Biochem. Sci. 13, 201-204
Fried, M., and Crothers, D. M. (1981) Nucleic Acids Res. 9, 6505-6525
Garner, M. M., and Jovanovic, A. (1981) Nucleic Acids Res. 9, 3047-3060
Harm, H., and Rupert, C. S. (1985) Mutat. Res. 133, 205-230
Harmeline, W. A., Gordon, L. K., Lindan, C. P., Graffstrom, R. H., Sharper, N. L., and Grossman, L. (1986) Nature 328, 504-509
Helene, C., and Maurizot, J. C. (1981) CRC Crit. Rev. Biochem. 10, 213-258
Husain, I., Sancar, G. B., Holbrook, S. R., and Sancar, A. (1987) J. Biol. Chem. 262, 13188-13197
Johnson, J. L., Hamm-Alvarez, S., Payne, C., Sancar, G. B., Rajagopalan, K. V., and Sancar, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2546-2550
Jorns, M. S., Sancar, G. B., and Sancar, A. (1984) Biochemistry 23, 2675-2679
Kiener, A., Husain, I., Sancar, A., and Walsh, C. (1989) J. Biol. Chem. 264, 13880-13887
Kim, S. T., and Sancar, A. (1991) Biochemistry 30, 8623-8630
Kim, S. T., Li, Y. F., and Sancar, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 900-904
Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488-492
Laemmli, U. K. (1970) Nature 227, 680-685
Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York
Lesser, D. R., Kurpioswki, M. R., and Jen-Jacobson, L. (1990) Science 250, 776-786
Li, Y. F., and Sancar, A. (1990) Biochemistry 29, 5598-5606
Malhotra, K., Baer, M., Li, Y. F., Sancar, G. B., and Sancar, A. (1993) J. Biol. Chem. 267, 2909-2914
Myles, G. M., Van Houten, B., and Sancar, A. (1987) Nucleic Acids Res. 15, 1227-1234
Pearlman, D. A., Holbrook, S. R., Prille, D. H., and Kim, S. H. (1985) Science 230, 1304-1309
Record, T. M., Jr., Lohman, T. M., and de Haseth, P. (1976) J. Biol. Chem. 251, 8608-8616
Rupert, C. S. (1962a) J. Biol. Chem. 236, 1478-1485
Sancar, G. B. (1985a) Proc. Natl. Acad. Sci. U. S. A. 82, 8858-8866
Sancar, G. B. (1985b) J. Biol. Chem. 260, 1478-1485
Sancar, G. B., Smith, F. W., and Heelis, P. (1989) Gene (Amst.) 64, 87-96
Sancar, G. B., Smith, F. W., and Heelis, P. F. (1987a) J. Biol. Chem. 262, 15457-15465
Sancar, G. B., Smith, F. W., Reid, R., Payne, G., Levy, M., and Sancar, A. (1987b) J. Biol. Chem. 262, 4783-4815
Sancar, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
Schulz, G. E., and Schritter, R. H. (1978) Principles of Protein Structure, Springer-Verlag, New York
Segel, I. H. (1975) Enzyme Kinetics, John Wiley and Sons, New York
Siebenlist, U., and Gilbert, W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 122-126
Takeda, Y., Kim, J. G., Caday, C. G., Steera, E. J., Ohlendorf, D. H., Anderson, W. F., and Matthews, W. B. (1986) J. Biol. Chem. 261, 8068-8076
Taylor, S. J., Garrett, D. S., Brocks, I. R., Swoboda, D. L., and Telser, J. (1990) Biochemistry 29, 8868-8875
Taylor, R. J., Jack, W. E., Robin, R. A., and Modrich, P. (1983) J. Biol. Chem. 258, 2820-2825
Weintraub, M. R., Altmann, E., Young, H., Begley, T., and Sancar, A. (1989) J. Am. Chem. Soc. 111, 9261-9265
Yasuhi, S., and Yasui, A. (1992) J. Biol. Chem. 267, 25464-25467