Identification of Chromophore Binding Domains of Yeast DNA Photolyase*

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Photolyases contain two chromophores, flavin plus either methenyltetrahydrofolate (MTHF) or 8-0H-5-deazaflavin (HDF). Amino acid sequence comparison reveals that all photolyases sequenced to date have extensive sequence homology in the carboxy-terminal half; in the amino-terminal region the folate and deazaflavin class enzymes are more homologous to other members of the same class. This modular arrangement of sequence homologies suggests that the amino-terminal half of photolyase is involved in MTHF or HDF binding whereas the carboxy-terminal half carries the flavin binding site. In this study we attempted to identify such structural domains of yeast photolyase by partial proteolysis and gene fusion techniques. Partial digestion with chymotrypsin yielded an amino-terminal 34-kDa fragment containing tightly bound MTHF and a carboxy-terminal 20-kDa polypeptide which lacked chromophore or DNA binding activity. However, a fusion protein carrying the carboxy-terminal 275 amino acids of yeast photolyase bound specifically to FAD but not to MTHF or DNA. We conclude that the amino-terminal half of yeast photolyase constitutes the folate binding domain and that the carboxy-terminal half carries the flavin binding site.

DNA photolyases catalyze the photocycloreversion of cyclobutane pyrimidine dimers in DNA utilizing the energy of 300–500 nm photons and thus reverse some of the biological effects of far UV (200–300 nm) radiation (see Sancar, 1990). All photolyases characterized to date contain two chromophores. One is FADH; (Sancar and Sancar, 1984; Eker et al., 1988), and the other is either 5,10-methenyltetrahydrofolate (MTHF; Johnson et al., 1988) or 8-hydroxy-5 deazaflavin (HDF; Eker et al., 1981, 1988). The FADH; chromophore is the catalytic cofactor in both classes whereas the second chromophores function as light harvesters owing to their high extinction coefficients in the near UV-visible range (Heelis et al., 1990; Eker et al., 1990). The genes for apoenzymes (phr) have been cloned and sequenced from four members of the folate class and three members of the deazaflavin class (see Sancar, 1990; Yasui et al., 1991). Comparison of the amino acid sequences of these photolyases has yielded an interesting pattern of conservation. The carboxy-terminal 190 amino acids are 52–75% identical among all photolyases, with no obvious systematic differences between the folate and deazaflavin classes whereas the amino-terminal halves of the enzymes display a much lower level of homology which appears to be class specific. Thus, a 100-amino acid stretch in the amino-terminal halves of photolyases from the eu bacteria Streptomyces griseus and Escherichia coli display only 11% homology; in contrast, when S. griseus and Halobacterium halobium (archebacterium) photolyases, both of which contain HDF, are compared, 36% identity is observed. Similarly, the amino-terminal regions of the MTHF-containing photolyases from E. coli and Saccharomyces cerevisiae (eukaryote) are 22% identical.

The above mentioned observations, coupled with the knowledge that photolyases from both classes make nearly identical contacts on DNA (Husain et al., 1987; Kiener et al., 1989; Baer and Sancar, 1989) and repair DNA by photoinduced electron transfer from FADH; (Okamura et al., 1991), have led to the general belief that the amino-terminal regions of photolyases are mainly responsible for binding of the light-harvesting antenna molecules whereas the carboxy-terminal half is involved in the two functions common to all photolyases: specific binding to FADH; and to Pyr=Pyr in DNA. However, comparison of photolyase sequences with those of other enzymes which bind flavin, deazaflavin, or tetrahydrofolate has revealed no obvious homologies to support this assignment (Takao et al., 1989; Sancar, 1990). Therefore, we decided to obtain direct experimental evidence for the proposed structural arrangement of photolyases. We used photolyase from S. cerevisiae for this purpose.

Partial proteolysis of yeast photolyase yields two fragments of 34 and 20 kDa, respectively. We found that the 34-kDa fragment, which originates from the amino-terminal half, contains the folate cofactor. The yield and instability of the carboxy-terminal 20-kDa fragment precluded extensive characterization. We obtained the carboxy-terminal domain in a stable form by fusing the last 275 amino acids of photolyase to the E. coli maltose-binding protein. The fusion protein bound FAD stoichiometrically. Neither the amino-terminal fragment nor the carboxy-terminal fusion protein bound DNA specifically. Thus, it appears that the amino- and carboxy-terminal halves of photolyases are involved in second chromophore (MTHF or HDF) and FAD binding, respectively, but the specific DNA binding requires interactions from both domains.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli CSR603 lacI (recA1, uvrA6, phr-1) containing the tac-PHRI plasmid pCB1241 was used as the source of yeast photolyase (Sancar et al., 1987). The same

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† The abbreviations used are: MTHF, methenyltetrahydrofolate; HDF, 8-hydroxy-5 deazaflavin; Pyr=Pyr cyclobutane, pyrimidine dimers; MBF, maltose-binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; THF, tetrahydrofolate.
strains were also used as the host for the fusion plasmid pMALcPHR1(C275). This plasmid was constructed by ligating the EcoRI-PstI fragment (from PCB1241) carrying base pairs 872-1668 of PHR1 into the same sites of pMALc vector (New England Biolabs). The resulting plasmid expresses a fusion protein containing the maltose-binding protein (MBP) joined to the carboxy-terminal 275 amino acids of yeast photolyase.

Materials—Single-stranded DNA cellulose, Blue Sepharose, trypsin inhibitor-agarose, chymotrypsin, and Tween 20 were purchased from Sigma. The chromatographic resins, amylase (New England Biolabs), P-100 (Bio-Rad), and Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) were obtained from the sources indicated. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim, restriction enzymes and T4 DNA ligase from Bethesda Research Laboratories, and Silica Gel G thin layer chromatography plates from Analtech.

Buffers—Lysis buffer consisted of 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10% (w/v) sucrose, and ammonium sulfate at 20% saturation. Buffer A contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, and 10 mM 2-mercaptoethanol. Buffer B contained 10 mM sodium phosphate, pH 6.8, 0.5 M NaCl, 1 mM sodium azide, and 10 mM 2-mercaptoethanol. Protease buffer consisted of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl2, and 5 mM dithiothreitol.

Preparation and Purification of Chymotryptic Fragments of Photolyase—Photolyase prepared as described (Sancar et al., 1987) and at 10.8 mg/ml in protease buffer (200 μl) was incubated with 5.4 μM chymotrypsin at 23°C for 15 min. The reaction mixture was then loaded onto a 1-ml trypsin inhibitor column equilibrated with Buffer A + 100 mM KCl. The column was washed with 7 ml of the same buffer, and 350 μl fractions were collected. The fractions were monitored for MTHF fluorescence at 470 nm, and those with fluorescence were combined and loaded onto a 1-ml single-stranded DNA cellulose column equilibrated with Buffer A + 0.1 M KCl. The flow rate was 0.2 ml/min. The column was washed with 5 ml of the same buffer, and then bound proteins were eluted with a step gradient of Buffer A + 0.1 M, 0.5, 0.75, 1.5, and 2.0 M KCl (1 ml each step). Fractions of 300 μl were collected and analyzed for protein content by SDS-PAGE and chromophore content by fluorescence spectroscopy.

Purification of MBP-PHR1(C275) Fusion Protein—A fresh colony of C. reinhardtii lacI/pAPgala/MALc-PHR1 was inoculated into 50 ml of Luria broth + ampicillin (200 μg/ml) and grown to late log phase as seed culture.

Results

Two Structural Domains of Photolyase—Yeast photolyase is composed of 565 amino acids (Sancar, 1985; Yasui and Langeveld, 1985). Digestion of the enzyme with chymotrypsin yields two stable intermediates of 34 and 20 kDa. During digestion the fluorescence of the enzyme (which is caused by the MTHF cofactor) increases steadily until digestion is complete, at which point the fluorescence intensity is about 2-fold greater than that of intact enzyme (Fig. 1) while the emission spectrum remains essentially the same. This suggests that although a quenching residue(s) has been removed from the vicinity of the folate, the nonpolar environment of the chromophore responsible for the red shift of emission (compared with MTHF in an aqueous environment) remains unchanged. Indeed, MTHF released from photolyase is converted rapidly at neutral pH to nonfluorescent 10-formyltetrahydrofolate (Johnson et al., 1988). Thus, it appears that MTHF must remain bound to one of the two chymotryptic fragments.

To find out which, after removal of chymotrypsin we loaded the sample onto a DNA-cellulose column which was developed with a salt gradient. The column fractions were analyzed by SDS-PAGE and fluorometry. The results are shown in Fig. 2. The 20-kDa fragment eluted in the wash and was associated with very little fluorescence, presumably resulting from free flavin. The 34-kDa fragment, in contrast, bound relatively tightly to the column, eluting at about 0.35 M KCl. The pattern of fluorescence intensity closely matched the elution profile from the column.

<FIG. 1. Relative folate fluorescence of yeast photolyase after chymotrypsin digestion. Yeast photolyase (10.8 μM) was digested with chymotrypsin (5.4 μM) for 15 min at room temperature in 200 μl of reaction mixture. Folate fluorescence of the enzyme prior to (---) and (----), after chymotrypsin digestion.>
The published sequence of yeast photolyase.

The profile of the 34-kDa band, and the fractions containing this fragment were free of any detectable uncleaved photolyase. Thus, we ascribe the fluorescence in these fractions to the 34-kDa polypeptide. Furthermore, since the excitation and emission spectra of the fluorescent species were those of enzyme bound MTHF, we conclude that the 34-kDa fragment carries the folate binding domain of DNA photolyase. This fragment may also be involved in DNA binding. However, this binding is nonspecific as evidenced by the fact that although the 34-kDa fragment is free of any detectable uncleaved photolyase.

Locations of the two domains—We sequenced the amino termini of both fragments and determined the amino acid composition of the 34-kDa fragment which was available in larger quantities because of better yield after proteolysis. The locations of the two fragments, based on these data, are shown schematically in Fig. 3. As is apparent from the figure the 34-kDa fragment originates from the amino-terminal and the 20-kDa fragment from the carboxyl-terminal half. There is some uncertainty regarding the carboxyl-terminal amino acid of both fragments. However, for the 34-kDa polypeptide the amino acid composition calculated from the known sequence (Sancar, 1985; Yasui and Langeveld, 1985), assuming no amino acid loss from the cleavage site between the hinge point separating the two fragments, is in a good agreement with the experimentally determined composition (data not shown).

Thus, we propose that the large fragment represents amino acids 16–326 of photolyase and may be referred to as the N(16–326) polypeptide.

Because of the lack of sufficient material we did not carry out amino acid analysis on the 20-kDa fragment. However, assuming that the protein is 20 kDa and starts at position 327, the cleavage at 505Y is the most likely possibility as it would yield a peptide with a calculated molecular mass of 21,080 daltons. The positioning of the two fragments in this manner is consistent with the prediction made from sequence comparison that the folate binding site is located in the amino-terminal domain. However, the relatively low yield and instability of the 20-kDa fragment precluded spectroscopic characterization of the carboxyl-terminal domain. We decided to express the carboxyl-terminal domain in a stable form, as a fusion protein, and investigate its properties.

MBP-PHR1(C275) Fusion Protein—An overproducing plasmid was constructed by joining the 3'-terminal 824 base pairs of PHR1 to the malE gene in the pMALc vector specifically designed for such constructs (Fig. 4). The fusion gene is under the control of a tac promoter and, upon induction with IPTG, a fusion protein of the expected size (~66 kDa) was overproduced.

Fig. 5 shows an SDS-PAGE analysis of the purification steps of the fusion protein. As is apparent from the figure, after IPTG induction the fusion protein constitutes about 1% of total cellular proteins and is easily separated by the amylose affinity column from all E. coli proteins except MBP. The material obtained in the last step (Fig. 5, lane 5) was fluorescent with excitation and emission spectra typical of FAD, suggesting that the fusion protein contained flavin. To obtain

![FIG. 2. Purification of the 34-kDa photolyase fragment and its association with folate fluorescence.](image2)

**Fig. 2**. Purification of the 34-kDa photolyase fragment and its association with folate fluorescence. Samples from the DNA-cellulose fractions were assayed for fluorescence at 470 nm (top panel) and were separated on 12% SDS-polyacrylamide gels which were silver stained and photographed (bottom panel). STD, molecular weight standards. **Lanes** 1–12, fractions after trypsin inhibitor column. Lanes 18–29, fractions eluted from the DNA-cellulose column with a step gradient of KCl from 0.25 to 2.0 M in Buffer A.

![FIG. 3. Locations of the chymotrypsin cleavage sites producing the 34- and 20-kDa photolyase fragments.](image3)

**Fig. 3**. Locations of the chymotrypsin cleavage sites producing the 34- and 20-kDa photolyase fragments. The cleavage site (shown with a broken arrow) at 505Y was predicted from the size of the carboxyl-terminal chymotryptic fragment. The sequences of amino-terminal 13 and 15 amino acids of the 34- and 20-kDa fragments, respectively, were determined experimentally and agree with the published sequence of yeast photolyase.

![FIG. 4. Restriction map of plasmid pMAL-PHR1.](image4)

**Fig. 4**. Restriction map of plasmid pMAL-PHR1. Construction of the plasmid is described under "Experimental Procedures." The vector contains the inducible P_tac promoter positioned to transcribe the malE-PHR1 gene fusion. Arrows indicate the direction of transcription.

![FIG. 5. Purification of the fusion protein as monitored by SDS-PAGE.](image5)

**Fig. 5**. Purification of the fusion protein as monitored by SDS-PAGE. The gel was stained with Coomassie Blue. **Lanes** 1 and 2 contain 200 μl each of uninduced cells, and cells induced with IPTG, respectively. **Lane** 3, cell-free extract (10 μl). **Lane** 4, peak fractions after amylose column (35 μl). **Lane** 5, peak fractions after blue Sepharose column (40 μl). **Lane** 6, molecular mass standards: bovine serum albumins (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa).
more definitive evidence fraction 4 was loaded onto a P-100 gel permeation column, and the fractions were analyzed on SDS-PAGE and fluorometrically. The results are shown in Fig. 6. As is apparent the fluorescence intensity matches the elution profile of the MBP-PHRI(C275) on Coomassie Blue-stained SDS-PAGE and not that of MBP. However, the fractions containing the fusion protein contain some minor contaminants, and therefore these data do not constitute a formal proof that MBP-PHRI(C275) is a flavoprotein. More definitive evidence was obtained from the flavin binding experiments described below.

**Binding of FAD to MBP-PHRI(C275)**—The excitation and emission spectra of the fusion protein were those of flavin. Furthermore, the fluorescence intensity at pH 2.6 was about 3-fold higher than the intensity at pH 7.7, identifying the chromophore as FAD and not FMN (Faeder and Siegel, 1973). Thin layer chromatography on silica gel confirmed this conclusion (data not shown). However, these analyses also revealed that FAD was present in grossly substoichiometric amounts (5–10%) in our purest protein preparations, precluding a rigorous exclusion of a minor flavoprotein contaminant in these fractions. We reasoned that if the fusion protein has a decreased affinity for FAD it may have not been saturated with the cofactor in vivo or might have gradually lost it during purification. To find out whether MBP-PHRI(C275) bound FAD specifically the fusion protein was incubated with a molar excess of FAD, and then unbound cofactor was removed by gel exclusion chromatography. Fig. 7 shows the relative fluorescence of the fusion protein before and after supplementing with FAD. From this figure it is estimated that only about 12% of the protein contained FAD before supplementation, in agreement with the fact that the absorbance spectrum of the fusion protein failed to yield an unambiguous flavin absorption at λ > 300 nm. In contrast, the reconstituted protein showed a diagnostic oxidized flavin absorption spectrum (λmax = 370 and 435 nm; Fig. 8). Using the protein concentration estimated from the calculated extinction coefficient of the fusion protein (ε390 = 130,000 M⁻¹ cm⁻¹) and the known extinction coefficient of free flavin at 435 nm (ε = 11,300), we calculate a stoichiometry of 2.0 µM FAD/1.6 µM fusion protein for the sample in Fig. 8B. Thus

![Fig. 6. Association of the fusion protein with flavin fluorescence.](image)

the carboxyl-terminal 275 amino acids of yeast photolyase are sufficient to bind specifically to FAD and constitute at least part of the flavin binding domain.

It must be noted, however, that the absorption spectrum of MBP-PHRI(C275) is like that of free FAD and does not show the vibrational "structure" observed with photolyase containing oxidized FAD (Payne et al., 1990, Jorns et al., 1990). The vibrationally resolved FAD spectrum in full-length photolyase and in other flavoproteins is caused by the nonpolar environment of the cofactor. Therefore, we must conclude that even though PHRI(C275) contains the chemical groups necessary for binding FAD specifically, other parts of the protein interact with this cofactor to provide the hydrophobic pocket and to maintain the flavin in the dihydro form in the native enzyme. In line with this conclusion the fusion protein saturated with FAD failed to bind Pyr−>Pyr-containing DNA specifically (data not shown). It has been shown that with full-length E. coli photolyase, the apoenzyme is incapable of binding DNA but acquires specificity upon reconstitution with FAD (Payne et al., 1990). Clearly, the carboxyl-terminal domain, even when bound to FAD, must be lacking some contacts essential for specificity.

**DISCUSSION**

Yeast photolyase contains two common cofactors, flavin and folate, albeit in uncommon forms: the flavin as FADH₂ and the folate in the form of MTHF. In the past decade sequences of many flavoproteins and of many enzymes which use folate cofactors have been published. A more restrictive form of the ββα supersecondary structure (the "Rossmann fold" (Rossmann et al., 1974)) has been posposed for the flavin
binding site of flavoproteins (Wierenga et al., 1986; van Beeumen et al., 1991), and conserved flavin binding sites have been proposed based on structures of flavoprotein reductases (Karplus et al., 1991; Porter, 1991). Sequence comparison of yeast or other photolyases by a number of alignment methods has failed to reveal any obvious sequence homology even with relatively liberal homology criteria for the flavin binding site. Similarly, sequence comparison of folate class photolyases (E. coli, Salmonella typhimurium, S. cerevisiae, Neurospora crassa) with tetrahydrofolate-binding enzymes failed to reveal any homology. Specifically, no homology was found to the proposed tetrahydrofolate binding site of thymidylate synthetases (Hardy et al., 1987); the methotrexate binding site of dihydrofolate reductases (Bzik et al., 1987); the 10-HCO-THF binding site of the human, rat, or S. cerevisiae trifunctional enzyme C1-tetrahydrofolate synthase (Hum et al., 1988); the 10-HCO-THF binding site of rat 10-HCO-THF synthase (Cook et al., 1991); or to folypolyglutamate synthetases from E. coli and Lactobacillus casei (Toy and Bagnar, 1990). Of special significance is the fact that the HPSLPL sequence, which is highly conserved in all 10-HCO-THF binding proteins including the 5,10-methenyltetrahydrofolate cyclohydrolase (Cook et al., 1991) is absent in yeast photolyase. Interestingly, an HPSL sequence is present in the carboxy-terminal half of E. coli photolyase (positions 288–291) but is not conserved even in the S. typhimurium enzyme which is 85% homologous to E. coli photolyase (Li and Sancar, 1991). Therefore, we think that this sequence plays no role in folate binding. It is reasonable to propose that since the function of the flavin and folate in photolyases involves an excited state and the excited states of molecules might be considered entirely different chemical species, the binding sites are radically different from other proteins which bind these cofactors in ground states. Perhaps equally relevant is the fact that in all other enzymes FAD and THF cycle between different stable redox states whereas this is not the case in photolyases.

In contrast to this lack of homology to other flavoproteins or enzymes with folate cofactors, photolyases from evolutionarily distant organisms have revealed a unique pattern of sequence conservation: a carboxy-terminal region highly conserved in all photolyases and an amino-terminal domain in which members of the two classes of photolyases (folate and deazaflavin) show unique stretches of homologies to members of their own class rather than an enzyme of the other class from an evolutionarily closer species (Takao et al., 1989; Sancar, 1990; Yasui et al., 1991). This observation has led to the provisional model that the carboxy-terminal half of photolyases carries the binding site for the two ligands common to all photolyases (FAD and DNA) whereas the amino-terminal half binds the ligand unique to each class. Two observations have provided some experimental support for the model. First, two tryptophan residues (W277 and W306) in the carboxy-terminal half of E. coli photolyase, which are conserved in all photolyases, have been found to be in close proximity with DNA and flavin, respectively (Li and Sancar, 1990; Li et al., 1991). Second, the single intron in the N. crassa gene is located at the junction of the two regions of homology (Yasui et al., 1991), suggesting dominant evolution for photolyases.

The experiments reported in this paper have borne out some of the predictions of the model but not others. The amino- and carboxy-terminal halves of yeast photolyase are capable of binding the folate and flavin chromophores respectively; however, the carboxy-terminal half is incapable of binding DNA specifically. In fact, the amino-terminal half has higher affinity to DNA-cellulose than the carboxy-terminal fragment. It is likely that the amino-terminal domain provides most of the contacts for nonspecific interactions whereas the specificity is accomplished by interaction of residues from both halves as well as interaction of the flavin cofactor with the substrate (Payne et al., 1990). Similarly, although the carboxy-terminal domain contains all the major contact sites for binding FAD, it apparently does not carry all the FAD-binding residues as evidenced by the fact that the absorption spectrum of FAD bound to the carboxy-terminal 275 amino acids of yeast photolyase in the fusion protein does not have the "structure" typically observed with flavins in nonpolar environments of the binding sites of flavoproteins including yeast photolyase (Sancar et al., 1987) or with flavins in nonpolar solvents. Thus, it appears that the amino-terminal half of the yeast photolyase must contribute some hydrophobic contacts to the flavin ring.

With these considerations, then, the following might be the evolutionary history of photolyases. A flavoprotein of 25 kDa capable of binding DNA was fused to a folate or a deazaflavin-binding protein of about the same size. The fusion provided additional contacts to keep the flavin in the active FADH₂ form and also increased the probability of repair by an incident photon by a factor of 3–4 (because of the high extinction coefficient of the second chromophore) and extended the range of usable light by 40–80 nm. The two domains coevolved so as to increase the efficiency of the binding of the chromophores and the efficiency of interchromophore energy transfer. As a consequence, the domains that were originally involved in binding one type of ligand acquired crucial contacts with the other ligands. Further studies with site-specific mutants and physical methods should help identify contacts essential for flavin reduction and substrate binding specificity.

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