Flavin adenine dinucleotide as a chromophore of the Xenopus (6-4)photolyase

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ABSTRACT

Two types of enzyme utilizing light from the blue and near-UV spectral range (320–520 nm) are known to have related primary structures: DNA photolyase, which repairs UV-induced DNA damage in a light-dependent manner, and the blue light photoreceptor of plants, which mediates light-dependent regulation of seedling development. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4)photoproducts] are the two major photoproducts produced in DNA by UV irradiation. Two types of photolyases have been identified, one specific for CPDs (CPD photolyase) and another specific for (6–4)photoproducts [(6–4)photolyase]. (6–4)Photolyase activity was first found in Drosophila melanogaster and to date this gene has been cloned only from this organism. The deduced amino acid sequence of the cloned gene shows that (6–4)photolyase is a member of the CPD photolyase/blue light photoreceptor family. Both CPD photolyase and blue light photoreceptor are flavoproteins and bound flavin adenine dinucleotides (FADs) are essential for their catalytic activity. Here we report isolation of a Xenopus laevis (6–4)photolyase gene and show that the (6–4)photolyase binds covalently to stoichiometric amounts of FAD. This is the first indication of FAD as the chromophore of (6–4)photolyase.

INTRODUCTION

Light is essential for life on Earth and organisms have evolved various methods for efficient utilization of light energy. Within the spectrum of sunlight, near-UV/blue light (320–520 nm) is utilized very efficiently and elegantly by two related systems. (i) In contrast to the many beneficial effects of solar light, the UV component is harmful to living cells, producing cytotoxic, mutagenic and carcinogenic lesions in DNA (1–3). This DNA damage can be repaired by near-UV/blue light by the DNA repair enzyme DNA photolyase (4,5). (ii) Numerous environmental factors influence plant development. Of these, light has an especially important role as a stimulus for many developmental processes. Blue light markedly affects growth and development of higher plants, including such phenomena as phototropism, chloroplast rearrangement, stomatal opening and inhibition of hypocotyl elongation. These responses are mediated by a blue light photoreceptor, cryptochrome (6).

The phenomenon of photoreactivation, the reduction of the lethal and mutagenic effects of UV radiation by simultaneous or subsequent irradiation with near-UV/blue light, has been identified in a variety of organisms. The enzyme responsible, CPD photolyase, binds to UV-damaged DNA and on absorption of a near-UV/blue light photon splits the cyclobutane ring, restoring the bases to their native form (7). In this reaction, the near-UV/blue light photon is used to excite FADH+ and flavin in the excited state then donates an electron to the CPD and thus FAD is essential for the reaction. The CPD photolyase gene has been isolated from 13 organisms and, on the basis of deduced amino acid sequence similarities, the genes have been grouped into two classes: Class I and Class II (8,9).

Light-dependent plant development, a complex process called photomorphogenesis, is controlled by the combined action of several photoreceptor systems (10). In higher plants there are at least three different families of photoreceptors: the red/far-red light receptor (phytochromes), the blue light receptor (cryptochrome; CRY) and a receptor for UV light. Although the best-studied signaling pathway in plants involves phytochrome, considerable research has been carried out in the past decade to characterize blue light perception and the signal transduction pathway (6). Recently, the first blue light photoreceptor in plants was characterized at the molecular level (11). This protein (CRY1) shows close homology to Class I CPD photolyase, although it exhibits no photolyase activity. CRY1 also binds FAD (12), suggesting that CRY1 mediates a light-dependent redox reaction similar to CPD photolyase.

Recently, we discovered another type of photolyase in Drosophila melanogaster that catalyzes the light-dependent repair of (6–4)photoproducts instead of CPDs and named this molecule

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(6-4)photolyase (13). Subsequently, the same enzymatic activity was identified in Xenopus laevis, Crotalus atrox (14) and Arabidopsis thaliana (15). It was previously thought that photoenzymatic reversal of (6-4)photoproducts was very unlikely for the following reasons. The formation of (6-4)photoproducts involves the transfer of the group at the C-4 position (-NH or -OH) of the 3' base of the dinucleotide to the C-5 position of the 5' base concomitant with the formation of a sigma bond between the C-6 of the 5' base and the C-4 of the 3' base. Even if an enzyme breaks the sigma bond joining the two adjacent pyrimidines, the bases would not be restored to their original forms. Thus, the mechanism of photoreactivation of (6-4)photoproduct is different from that of CPD (16). The gene encoding (6-4)photolyase was cloned from Drosophila (17). Unexpectedly, the deduced amino acid sequence of (6-4)photolyase was found to be similar to the Class I CPD photolyase and CRY1. Thus we call these proteins the DNA photolyase/blue light photoreceptor family. Based on the amino acid sequence similarities, we set out to clone the Xenopus (6-4)photolyase cDNA by polymerase chain reaction (PCR). Here, we describe the isolation and characterization of a cDNA encoding (6-4)photolyase from X. laevis. We show that (6-4)photolyase binds FAD similarly to other members of the DNA photolyase/blue light photoreceptor family, although CPD and (6-4)photolyase operate by different mechanisms.

MATERIALS AND METHODS

Preparation of crude cell extracts from Xenopus oocytes

Isolated oocytes were homogenized in 1 ml buffer containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). To the homogenate was added 1 ml ice-cold solution containing 10 mM Tris–HCl, pH 8.0, 5 mM DTT, 25% sucrose and 50% glycerol. After mixing gently, 160 μl 5 M NaCl was added, mixed for 20 min at 4°C and then centrifuged at 15 000 g for 20 min at 4°C. The supernatant was used as crude extract. Aliquots of 2 μg crude extract were used for gel shift assays as described previously (13,18).

Isolation of Xenopus (6-4)photolyase cDNA clone

Unless otherwise noted, all RNA and DNA manipulations were carried out using standard techniques (19). To prepare the probe for hybridization, PCR was carried out with Xenopus ovary cDNA and degenerate oligonucleotides (64PRN1, 5'–T[A/G/C/T]G[C/A/G/T]TTGT[C/A/G/T]G[A/G/T]T[T/C]TA-3'; 64PRC1, 5'–CC[T/C]TC[T/C]TCCC[C/A/G/T][G/C][A/T][A/G/T]ATCCA-3'; 64PRC2, 5'–TG[A/G/T][G/T]GCC[A/G/C/T]AG[A/G/T][G/A/T]TATCCA-3') based on regions conserved between CPD photolyase and (6-4)photolyase (17). Two rounds of PCR were carried out. The first round was carried out with primers 64PRN1 and 64PRC1 and aliquots of the first round PCR product were used for the second round of PCR with primers 64PRN1 and 64PRC2. A 160 bp amplified product was sequenced and found to be related to Drosophila (6-4)photolyase and this was used to screen a Xenopus oocyte cDNA library (Clontech). Four positive clones were isolated, the longest of which was recloned into pUC19 and sequenced on both strands by the standard dideoxy chain termination method (19).

Purification of Xenopus (6-4)photolyase

Plasmid pGEX-XI64PR was constructed by inserting the coding sequence of Xenopus (6-4)photolyase cDNA into BamHI/EcoRI-digested pGEX-4T-2 (Pharmacia) and used for transformation of Escherichia coli SY2(uvrA+, recA+, phr-). Transformed cells were grown at 26°C in 3 l LB medium containing 150 mg/l ampicillin (19) until an A600 of 0.9–1.0 was reached. Expression was induced by addition of 0.1 mM isopropyl β-d-thiogalactopyranoside (IPTG) and growth was continued at 26°C for 9 h. Cells were harvested by centrifugation and resuspended in 50 ml phosphate-buffered saline (PBS). Cell extract was prepared by sonication of the cell suspension, followed by centrifugation at 15 000 g for 60 min at 4°C. The supernatant (Fraction I) was applied to a glutathione–Sepharose column (10 ml). Purification using glutathione–Sepharose and removal of glutathione S-transferase (GST) by cleavage with thrombin were performed according to the manufacture’s instructions (Pharmacia). The eluate from the glutathione–Sepharose column (Fraction II) was treated with thrombin and the thrombin-cleaved sample (Fraction III) was applied to a UV-irradiated DNA affinity column equilibrated with 50 mM phosphate buffer, pH 7.5, containing 50 mM KCl. After washing with 15 ml elution buffer (50 mM phosphate buffer containing 2 M KCl), the eluted sample was concentrated using Centricon 50 (Amicon) and elution buffer was replaced with equilibration buffer. Finally, 1 ml protein solution was obtained (Fraction IV). Starting from 3 l E. coli culture, 700 mg cell extract (Fraction I), 5 mg protein eluate from glutathione–Sepharose (Fraction II) and 1.4 mg UV-irradiated DNA affinity column purified protein (Fraction IV) were recovered. The concentration of protein was determined with a Bradford assay kit (BioRad). The UV-irradiated DNA affinity column was prepared as described previously (21).

Photoreactivation treatment and ELISA were carried out as described previously (13,17). For ELISA and for the repair assay using a (6-4)photoproduct-containing oligonucleotide, 0.1 and 1 μg Fraction III were used respectively.

Preparation of a DNA fragment containing (6-4)photoproduct

The deoxyoligonucleotide 28mer substrate [d(CCCGAAAGA-CAGT[6-4]TAAACCGCGAAGCG)] containing a (6-4)photoproduct at the central TT site was constructed by ligation of a 64mer [d(CAGTTTGGTGTATTGTCACTGCGGGTGTCTTTATACAA)] and a 10mer [d(CCCGAAAGA) and d(CACGCAAACG)] after annealing with a 32mer [dTTCGTTTGGTGTATTGTCACTGCGGGTGTCTTTATACAA)] using the procedure described previously (22). Resultant duplex DNA was purified by gel electrophoresis and labelled with [γ-32P]ATP (3000 Ci/m mol) and T4 polynucleotide kinase. The labelled DNA (5 × 104 c.p.m.) was mixed with purified Xenopus recombinant protein (1 μg Fraction III) and exposed to fluorescent lamps for 30 min. After irradiation the DNA was then extracted with phenol/chloroform and precipitated with ethanol. The DNA was digested with Hpal (10 U) and separated on 10% polyacrylamide sequencing gels.

Chromophore isolation from recombinant Xenopus (6-4)photolyase and reconstitution of E. coli CPD photolyase

Recombinant Xenopus (6-4)photolyase purified with a UV-irradiated DNA affinity column (Fraction IV) was denatured at pH 3.0 by heating at 65°C for 10 min. The released chromophore was recovered by filtering out the denatured protein using Microcon 30 followed by Microcon 3 (Amicon), Escherichia coli photolyase apoenzyme was prepared as described previously (23). Reconstitut-
tion of enzymatic activity with either authentic FAD or chromophore isolated from *Xenopus* (6-4)photolyase was conducted by incubating the apoenzyme (400 μM) with the indicated chromophore (40 μM) at 10°C for 24 h (24). The concentration of chromophore was based on the absorbance at 450 nm (ε_{450} = 1.12 × 10^{4} M^{-1} cm^{-1}).

**CPD photoreactivation assay**

The oligo(dT)20 substrate containing CPDs was prepared by acetone-photosensitized irradiation under a cold nitrogen atmosphere. Since CPD has no absorption at 265 nm, the increase in absorbance at 265 nm was used to estimate CPD repair (25). For the photoreactivation assay, enzyme (4 μM) was mixed with substrate (30 μM) in 200 μl buffer containing 50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 5 mM DTT and 10% glycerol. The reaction mixture was placed in a cuvette, deoxygenated under a gentle stream of cold nitrogen and exposed to filtered camera flashes (340 nm cut-off filter) prior to irradiation with photoreactivating light (350–450 nm) at 10°C.

**RESULTS**

**Cloning of *Xenopus* (6-4)photolyase**

Previously we have shown that in *Drosophila* DNA photolyase genes are expressed at a very high level in the ovary and its translated products are stored in eggs (9,17). This suggests that ovary is a good candidate for testing (6-4)photolyase activity to photoreceptor family (see Materials and Methods). A 160 bp regions conserved between the DNA photolyase/blue light a factor which binds specifically to (6-4)photoproduct in the same gel shift assay as reported previously (17). We detected (6-4)photoproduct in cell extracts from genes are expressed at a very high level in the ovary and its cloning of *Xenopus* oocyte cDNA library and we identified a 2.5 kb cDNA done. Sequencing of the cDNA clone revealed the presence of a single long open reading homologue and 20-24% identity to the class Drosophila (6-4)photolyase and its human homologue (34). We tested binding activity specific for (6-4)photoproduct in *Xenopus* ovari cells extracts (Fig. 1). To generate a probe to screen a *Xenopus* cDNA library, we used PCR with primers based on regions conserved between the DNA photolyase/blue light photoreceptor family (see Materials and Methods). A 160 bp DNA fragment amplified from cDNA prepared from *Xenopus* ovari cells was used as a probe to screen a *Xenopus* oocyte cDNA library and we identified a 2.5 kb cDNA clone. Sequencing of the cDNA clone revealed the presence of a single long open reading frame capable of encoding a protein of 526 amino acids, corresponding to a predicted molecular mass of 60.6 kDa. The sequence of the cDNA predicted a protein that showed 58–54% amino acid identity to *Drosophila* (6-4)photolyase and its human homologue and 20–24% identity to the class I CPD photolyase and the blue light photoreceptor over the entire protein (data not shown). Thus the cloned cDNA is a member of the DNA photolyase/blue light photoreceptor family.

**Expression of the cDNA in *E.coli***

To verify that the isolated cDNA clone encodes (6-4)photolyase, we measured enzymatic activity of the recombinant protein expressed in *E.coli*. The cDNA was inserted into a prokaryotic expression vector designed to produce a GST fusion protein and named pGEX-X164PR. *Escherichia coli* does not photoreactivate (6-4)photoproduct and thus would show increased resistance to UV light on expression of (6-4)photolyase in the presence of photoreactivating light. As expected, the plasmid pGEX-X164PR conferred light-dependent UV resistance on the apoenzyme (400 μM) with the indicated chromophore (40 μM) at 10°C for 24 h (24). The concentration of chromophore was based on the absorbance at 450 nm (ε_{450} = 1.12 × 10^{4} M^{-1} cm^{-1}).

**Identification of the chromophore**

Purified *Xenopus* (6-4)photolyase was a yellow colour and had an absorption spectrum resembling those of many flavoproteins (Fig. 3). The chromophore was released by heat or acid treatment of *Xenopus* (6-4)photolyase, indicating that it was non-covalently bound to the enzyme. The absorption spectrum of the free chromophore was identical to that of fully oxidized flavin adenine dinucleotide (FAD) (Fig. 3). The identity of the chromophore as FAD was also suggested by thin layer chromatography and an increase in fluorescence intensity on acidification (data not shown). To determine that this chromophore was indeed FAD, we reconstituted *E.coli* CPD photolyase activity from its apoenzyme and the chromophore isolated from *Xenopus* (6-4)photolyase. *Escherichia coli* CPD photolyase requires bound FAD as a catalytic cofactor. The holoenzyme (FAD–bound *E.coli* photolyase) showed high affinity for CPDs (Fig. 4A, lane 5) and repaired them in a light-dependent manner, although the apoenzyme had no affinity for CPDs (Fig. 4A, lane 2) and no photocatalytic activity (Fig. 4B) (23). When the apoenzyme was mixed with the chromophore isolated from *Xenopus* (6-4)photolyase, the resulting reconstituted photolyase restored both binding (Fig. 4A, lane 3) and the ability to repair CPDs (Fig. 4C) (23).
and photocatalytic activity (Fig. 4B). The molar ratio of FAD released from \textit{Xenopus} (6-4)photolyase relative to its apoprotein was 0.95 as calculated from the coefficients of the apoprotein ($\varepsilon_{280} = 1.30 \times 10^5$ M$^{-1}$cm$^{-1}$). The excitation coefficient for the \textit{Xenopus} (6-4)photolyase apoprotein was calculated using the number of tryptophan (18; $\varepsilon_{280} = 5800$ M$^{-1}$cm$^{-1}$) and tyrosine (18; $\varepsilon_{280} = 1405$ M$^{-1}$cm$^{-1}$) residues determined from the DNA sequence of the \textit{Xenopus} (6-4)photolyase gene. Together, these results show that \textit{Xenopus} (6-4)photolyase binds FAD.

**DISCUSSION**

CPDs and (6-4)photoproducts are the two major classes of cytotoxic, mutagenic and carcinogenic photoproducts produced in DNA when cells are irradiated with UV light (2,3,5). These lesions are repaired by the nucleotide excision repair pathway, although CPDs are repaired less efficiently than (6-4)photoproduct. CPDs are most efficiently repaired by DNA photolyase (4). It has long been believed that CPDs are the only substrate for photolyase. As a consequence, it has become common practice to expose UV-irradiated cells to photoreactivating light (350-450 nm) to study the effects of (6-4)photoproduct. Any residual mutagenic or cytotoxic effects remaining following photoreactivation are ascribed to (6-4)photoproduct (3). In contrast to the general belief that CPDs are the only substrate for photolysis, we discovered a new type of photolysis in \textit{D.melanogaster} which catalyzed light-dependent repair of (6-4)photoproduct [(6-4)photolyase] (13). In this paper we have identified the \textit{Xenopus} (6-4)photolyase gene. This is the first molecular description of a (6-4)photolyase gene in a vertebrate. An enzymatic activity of (6-4)photolyase has also been detected in the rattlesnake and a higher plant (14,15), indicating that (6-4)photolyase might be widely distributed among present organisms. Thus, interpretations of the effects of photoreactivation on UV-irradiated cells reported previously should be reconsidered. In frog cells (ICR 2A) (6-4)photoproduct was removed rapidly from DNA of UV-irradiated cells following photoreactivation (26). This might show photoreactivation of (6-4)photoproduct in ICR 2A cells, although it was interpreted...
reduced FAD is the active form and (6-4) photoproduct is repaired by the photoreceptor, oscillation of FAD between its different redox states determines the response wavelength for each plant cell (12). Photoreduction of purified Xenopus (6-4) photolyase led to an increase in the capability for excision of (6-4) photoproduct (26).

We have demonstrated that the (6-4) photolyase is a flavoprotein, similar to other members of the DNA photolyase/blue light photoreceptor family. Each member of this family utilizes light energy through FAD in various reduced forms. In CPD photolyase, FADH$^-$ is the active form which donates an electron to the CPD, resulting in splitting of the cyclobutane ring (7). In the blue light photoreceptor, oscillation of FAD between its different redox states determines the response wavelength for each plant cell (12). Photoreduction of purified Xenopus (6-4) photolyase led to activation of repair activity (data not shown), indicating that reduced FAD is the active form and (6-4) photoproduct is repaired by electron donation, as is the case for CPD. A possible pathway for repair by (6-4) photolyase was proposed previously (16), in which binding of (6-4) photolyase to DNA was suggested to thermally convert the (6-4) photoproduct to its oxetane intermediate and then electron transfer from excited FAD to the intermediate restored the original form. Our results are consistent with this model. Complete understanding of the repair mechanism must await further characterization of (6-4) photolyase.

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