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Influence of Ultraviolet-C on Structure and Function of *Synechococcus* sp. PCC 7942 Photolyase

L. W. Zhang, M. Li, and Q. Y. Wu*

Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, P. R. China; fax: 86-10-627-81825; E-mail: qingyu@tsinghua.edu.cn

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Abstract—In this work, an over-expressed cyclobutane pyrimidine dimer (CPD) photolyase of *Synechococcus* sp. PCC 7942 was used to investigate UV-C (ultraviolet irradiation of C-region) influence on photoreactivation. *In vivo* photoreactivation experiments indicated that the survival rate decreased from 100 to 2.6% when the UV-C flux was increased from 1.1 to 68.5 μW/cm^2. It seemed that the photolyase was easily inactivated at UV-C intensities ≥25.5 μW/cm^2. Spectrometric analysis indicated that tertiary structure of the photolyase changed evidently when the UV-C fluxes were ≥25.5 μW/cm^2, while the secondary structure was almost unchanged even at 170 μW/cm^2. Band shift assay indicated that catalytic activity of the photolyase was impaired at fluxes ≥25.5 μW/cm^2, but no significant influence on DNA-binding activity was observed. These results suggest that photoreactivation is efficient at UV-C fluxes ≤25.5 μW/cm^2, but would be impaired by intense UV-C irradiation due to structure changes of the photolyase.

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Key words: circular dichroism (CD) spectrum, cyclobutane pyrimidine dimer (CPD) photolyase, fluorescence spectrum, *Synechococcus* sp. PCC 7942, ultraviolet resistance

There can be no doubt that both the processes involved in the origin of life and the events that took place during the early evolution of life on the Earth were strongly affected by ultraviolet (UV) radiation from the Sun. It is well known that nucleic acid is sensitive to UV radiation, which requires some internally consistent protection for the origin and early evolution of life on the Earth [1]. Photoreactivation is a simple but efficient DNA repair pathway, in which process photolyases use blue or near-UV light to repair UV-induced DNA lesions—mainly some pyrimidine (Pyr) dimers. First, the enzyme binds the Pyr dimers on DNA in a light independent manner, then utilizes light energy to convert them to the original two pyrimidines, and dissociates from the repaired DNA [2]. Photorepair is proved more important than excision repair for UV-induced DNA lesions in *Synechocystis* sp. PCC 6803 [3, 4].

In view of the special capability to repair UV-induced DNA lesions, it is of great significance to investigate UV resistance of the photolyase in order to clarify how some organisms survive intense UV radiation, especially during the origin of life on Earth. As far as we know, there is no report on UV-C (ultraviolet irradiation of C-region) influence on photoreactivation, though photolyases have been investigated structurally and functionally in a wide range of organisms [2-5].

In this work, *Synechococcus* sp. PCC 7942 photolyase was overexpressed in *Escherichia coli* BL21(DE3). It was investigated under varying doses of UV-C irradiation *in vivo* and *in vitro*, aiming at elucidating the influence of UV-C on photoreactivation.

MATERIALS AND METHODS

Materials. Restriction enzymes, Pfu polymerase, and ligase were obtained from Takara Biotech (China). Low molecular weight protein marker, DNA marker, *E. coli* BL21(DE3), and Ni-chelating HP column were purchased from Dingguo Biotech (China). Plasmid pET-28a(+) was purchased from Novagen (USA).

Overexpression and purification of *Synechococcus* sp. PCC 7942 photolyase. The photolyase gene (*phr*) was

Abbreviations: CPD) cyclobutane pyrimidine dimer; IPTG) isopropyl-thio-β-D-galactoside; UV-A, UV-B, UV-C) ultraviolet irradiation of A-, B-, and C-region, respectively.

* To whom correspondence should be addressed.

amplified from a genomic library of *Synechococcus* sp. PCC 7942 by PCR using the following primers tagged with NcoI and XhoI restriction sites: (up) 5′-GAGACCATGGCGGCTCCGATTCTGTTTTGG-3′ and (down) 5′-GAGACTCGAGTGAATCGGGCTCAGCCTCAG-3′. The resultant fragments were digested with NcoI/XhoI restrictases and cloned into the NcoI/XhoI sites of pET-28a vector, yielding a plasmid pET-28a/phr, which was confirmed by DNA sequencing.

The photolyase was overexpressed by isopropyl-thio-β-D-galactoside (IPTG) induction of the *E. coli* BL21 cells transformed with pET-28a/phr and purified with the Ni-chelating HP column according to the product manual. Purified fractions were combined and concentrated using a YM-10 ultrafiltration apparatus (Millipore, USA), and the buffer was changed to buffer A (5 mM sodium phosphate, pH 7.4) or buffer B (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 125 mM NaCl, and 4% glycerol) [6]. Protein concentration was determined using the Coomassie Brilliant Blue method [7].

Light sources. A germicidal lamp (254 nm) was used to provide UV-C irradiation. The UV flux was regulated by changing the distance between the lamp and sample. A General Electric black light (λ_{max} = 365 nm, 654 μW/cm^2) was used for UV-A (ultraviolet irradiation of A-region) photoreactivation. The light intensity was measured using a UV radiometer equipped with specific UV sensors sensitive at 254 and 365 nm, respectively (Beijing Electro-Optical Equipment Factory, China).

***In vivo* photoreactivation and UV resistance of the photolyase.** The *in vivo* photoreactivation study was carried out using *E. coli* cells transformed with pET-28a/phr or pET-28a, and the cells transformed with pET-28a/phr were induced with 0.3 mM IPTG to overexpress the photolyase. Aliquots (50 μl) of 10^4 times dilutions of log-phase cultures of the *E. coli* cells were plated on LB medium supplemented with 50-μg/ml kanamycin. With lid removed, the plates were irradiated with UV-C at different flux rates from 0 to 170 μW/cm^2 for 15 min. For photoreactivation of irradiated cells, plates were placed under black light for 30 min. Control plates were kept in the dark after the same UV-C irradiation. Then all the plates were cultivated at 37°C for 20 h in the dark, and colonies on each plate were counted. Three parallel experiments were performed and the colony numbers for the same sample were averaged.

Spectrometric analysis. Far-UV circular dichroism (CD) spectra were measured on a J-600 spectrophotometer (Jasco, USA) using a 1 mm quartz cell. Spectra were collected from 250 to 200 nm with a scanning rate of 50 nm/min and a bandwidth of 0.5 nm with a time response of 2 sec. CD spectra were obtained as an average of 3 scans.

Fluorescence measurements were performed on an F-2500 FL spectrophotometer (Hitachi, Japan). According to Zhang [8] and Cleiner [9], intrinsic tryptophan fluorescence emission spectra and FAD fluorescence emission spectra were measured in a scan range from 300 to 390 nm and from 480 to 590 nm at an excitation wavelength of 295 and 460 nm, respectively. The buffer alone spectra were subtracted from the corresponding sample spectra. The concentration of the photolyase for all spectrometric measurements was kept at ~0.2 mg/ml in buffer A.

Band shift assay. DNA-binding and catalytic activity of the photolyase after varying doses of UV-C irradiation were evaluated using band shift assay performed in 2% agarose gel. A 237-bp PCR product containing 15 consecutive thymine bases was prepared as DNA substrate in the band shift assay. It was irradiated with UV-C (254 nm, 1100 μW/cm^2) for 60 min to produce pyrimidine dimers. The dimer formation was monitored by the decrease of the monomer absorption band at 264 nm [10]. DNA concentration was valuated by absorbance at 260 nm measured on a Ultrospec 2000 UV/Vis spectrophotometer (Pharmacia, Sweden).

Aliquots (40 μl) of the photolyase in buffer B were irradiated with varying fluxes of UV-C for 15 min. Irradiated photolyase samples (10 μl, ~200 ng) were mixed with 10 μl DNA or UV-C irradiated DNA (UV-DNA) (~100 ng) and placed at 4°C for 30 min. The mixtures were run on agarose gel directly or after a sequential photoreactivation with black light for 20 min. This experiment was carried out in the dark.

RESULTS

Overexpression and identification of the photolyase. The *Synechococcus* sp. PCC 7942 photolyase was overexpressed in *E. coli* BL21 and analyzed by SDS-PAGE. A protein band of approximately 55 kD was overexpressed by IPTG induction (Fig. 1), similar to the size (54.47 kD) predicted from its amino acid sequence (GenBank accession No. NC007604). The photolyase was identified by a photoreactivation experiment (data not shown).

Influence of UV-C on photoreactivation *in vivo*. For both pET-28a/phr and pET-28a transformed cells, UV-A irradiation could greatly enhance the survival rate, suggestive of photoreactivation in these cells (Fig. 2). After identical UV-C radiation and a sequential UV-A photoreactivation, the survival rate of the pET-28a/phr transformed cells was much higher than that of pET-28a transformed cells, suggesting that the increment of photolyase enhanced the photorepair activity for cyclobutane pyrimidine dimers (CPDs). Curves *4* and *5* showed that there was almost no increment on survival rate for the cells containing more photolyase, indicating that the photolyase could not perform its function in the dark. It is evident that the survival rate was reduced with increasing UV flux, which was only 2.8% after UV-C irradiation at

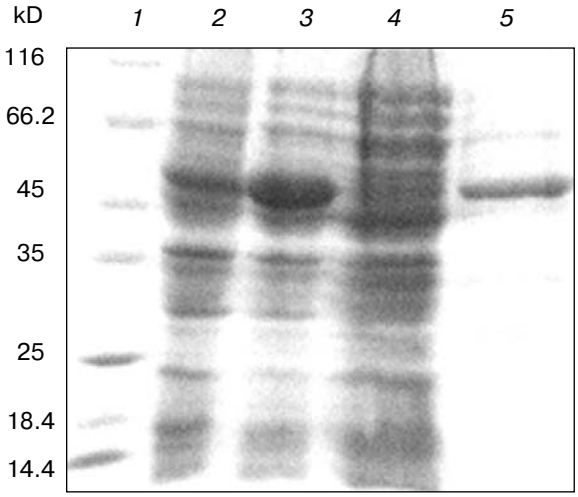

Fig. 1. SDS-PAGE analysis of the proteins expressed in *E. coli* BL21 on IPTG induction. Lanes: *1*) molecular weight markers; *2*) pellet fraction of pET-28a/phr transformed cells; *3*) pellet fraction of pET-28a/phr transformed cells induced by IPTG; *4*) supernatant fraction of the IPTG induced cells; *5*) purified protein.

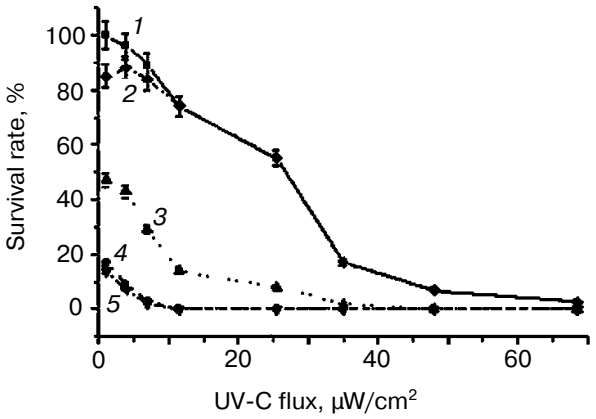

Fig. 2. Photoreactivation of UV-induced DNA lesions in *E. coli* cells. Curves: *1*, *4*) *E. coli* cells transformed with pET-28a/phr after photorepairing and without it, respectively; *3*, *5*) *E. coli* cells transformed with pET-28a after photorepairing and without it, respectively. Curve *2* was obtained by subtracting curve *4* from curve *1*. The survival rates were determined by the colony number of each samples divided by the colony number of the control. The error bars are the range for three replicates.

68.5 μW/cm^2. At 170 μW/cm^2 UV-C, no *E. coli* cell could survive, even prolonging the culturing time to 40 h or photorepairing cells simultaneously with UV-C irradiation (data not shown), indicating that the photolyase was inactivated by intense UV-C irradiation. To investigate the influence of UV-C irradiation on photoreactivation, curve *2* was obtained by subtracting curve *4* from curve *1*, which shows the photoreactivation changes with increasing UV-C intensity. When UV-C intensity was increased from 11.5 to 25.5 μW/cm^2, the survival rate decreased from 74 to 55%, and when the UV intensity was increased from 25.5 to 35 μW/cm^2, the survival rate decreased from 55 to 17%. The survival rate changed a little when the UV-C intensity was increased from 35 to 68.5 μW/cm^2. Considering the changes on curve *2*, it seemed that the photolyase was easily inactivated at UV-C intensities ≥25.5 μW/cm^2.

Structural changes after UV-C irradiation. Far-UV CD was used to probe secondary structural changes in the photolyase after varying doses of UV-C irradiation. As shown in Fig. 3, the CD spectra of all the samples exhibit a similar shape with two minimums at 208 and 216 nm, a feature typical of α-helix- and β-sheet-dominant proteins [8]. It is indicated that the photolyase was almost unchanged in secondary structure under the experimental UV conditions.

As a probe of tertiary structure change, fluorescence spectra of the photolyase were recorded after varying doses of UV-C irradiation. Figure 4a shows that the fluorescence maximum was ~332 nm, indicating the emitting tryptophan residues might be in a shielded hydrophobic environment. After UV-C irradiation, the fluorescence intensities increased, suggestive of a decreased level of quenching due to increased motional flexibility for the emitting tryptophans, which in the native state might be highly quenched by some adjacent groups. Meanwhile, there is a small red shift (~3 nm) after UV-C irradiation at 170 μW/cm^2, suggesting that the emitting tryptophans become increasingly exposed due to the loosened structure. Considering the invariable fluorescence maximum, the tertiary structure of the photolyase might change only slightly under UV-C irradiation ≤25.5 μW/cm^2. In Fig. 4b, after UV-C irradiation at 170 μW/cm^2, the fluorescence intensity enhanced more than fivefold, probably due to the release of FAD from its binding site. At

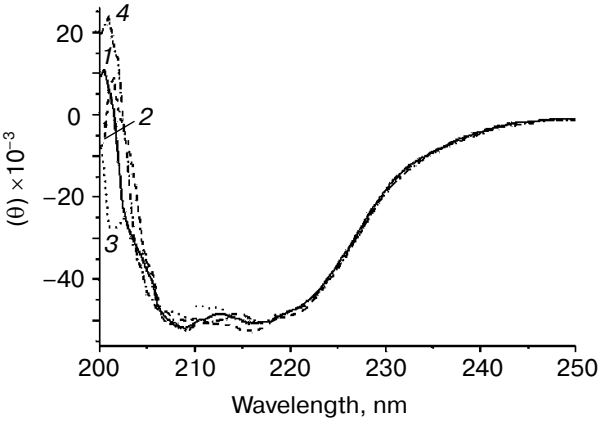

Fig. 3. CD spectra of the photolyase after varying doses of UV-C irradiation. Curves: *1*) control (not irradiated with UV-C); *2-4*) UV-irradiated with intensities of 11.5, 25.5, and 170 μW/cm^2, respectively.

11.5 μW/cm^2, the fluorescence spectrum was almost the same as that of the control, indicating that the environment near FAD was little affected by UV-C irradiation at so low a flux.

Influence of UV-C on enzyme activity of purified photolyase. CPD photolyase can specifically bind to Pyr dimers, the coenzyme FADH$^-$ transfers an electron to Pyr dimers using excitation energy; the 5–5 and 6–6 bonds of the cyclobutane ring are split to generate a Pyr and Pyr$^-$, the latter donates an electron back to the flavin cofactor to regenerate FADH$^-$ and the enzyme dissociates from DNA [2]. The control sample mixed with non-UV irradiated DNA and photolyase showed the same band as that of UV-DNA, while the samples mixed with UV-DNA and photolyase showed an additional DNA band other than the UV-DNA band, suggesting that a UV-DNA–photolyase complex was formed due to the specific binding activity of the photolyase (Fig. 5a). It was observed that the binding activity was almost not affected after varying doses of UV-C irradiation. However, the catalytic activity was affected by increasing UV-C irradiation. At UV-C fluxes ≥25 μW/cm^2, the photolyase–DNA complexes remained unchanged after UV-A irradiation (Fig. 5b). The photolyase could not dissociate from the DNA substrate, even prolonging the photoreactivation time to 40 min (data not shown), suggesting that the photolyase could not repair the DNA lesions after severe UV-C irradiation (≥25 μW/cm^2).

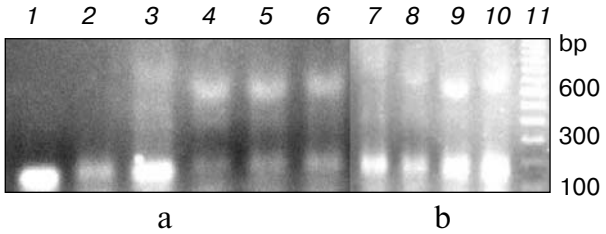

Fig. 5. Enzyme activity assay of the photolyase. a) Binding activity assay: *1*) non-UV irradiated DNA; *2*) UV-DNA; *3*) non-UV irradiated DNA and native photolyase; *4*) UV-DNA and native photolyase; *5*, *6*) UV-DNA and UV-C irradiated photolyase (the UV-C intensity was 25.5 and 170 μW/cm^2, respectively). b) Photorepair activity assay after UV-A irradiation for 20 min: *7*) UV-DNA and native photolyase; *8*-*10*) UV-DNA and UV-C irradiated photolyase (the UV-C intensity was 11.5, 25.5, and 170 μW/cm^2, respectively); *11*) 100 bp DNA ladder.

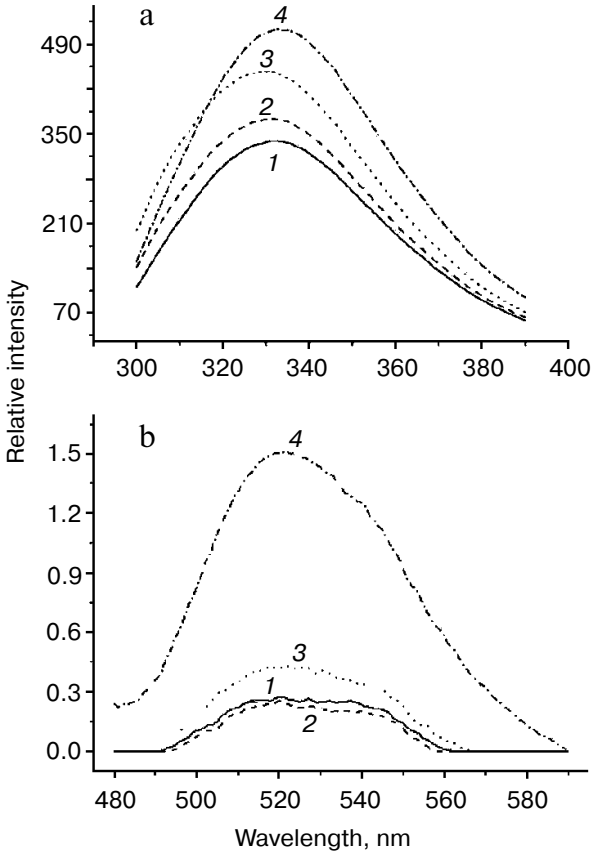

Fig. 4. Fluorescence emission spectra of tryptophan (excited at 295 nm) (a) and FAD (excited at 460 nm) (b) in the photolyase after varying doses of UV-C irradiation. Curves: *1*) control (not irradiated with UV-C); *2*-*4*) UV-irradiated with intensities of 11.5, 25.5, and 170 μW/cm^2, respectively.

DISCUSSION

There is little solar UV-C radiation reaching the Earth now, but it was severe during the origin of life and decayed later with increasing ozone layer. UV-induced DNA damage was probably even more severe before the formation of the ozone shield. Therefore, selective pressure existed to develop a self-defense system such as DNA photolyases [1]. In this work, we selected *Synechococcus* sp. PCC 7942 photolyase to investigate the influence of UV-C on photoreactivation.

The control cells were UV-sensitive at a relatively low dose (1.1 μW/cm^2, 20 min), compared with the cells photoreactivated by UV-A. This is in agreement with previous reports [3, 4] and indicates that photorepair is more important than other repair pathways such as excision repair for UV-induced DNA lesions. Up-regulation of the photolyase expression has been found in several species of organisms [11]. Our results suggest that the up-regulated expression of the photolyase is of great importance to help organisms survive intense UV radiation (Fig. 2).

Previous study indicates that UV-B (ultraviolet irradiation of B-region)-photodamaged aldehyde dehydrogenase with alterations in its quaternary structure, but no significant change was noticed in the peptide chain conformation. Under this condition, the enzyme could still perform its protective function in preventing aggregation of photolabile proteins [12]. In the present work, we

found that UV-C irradiation could result in tertiary structure changes in the photolyase, but there was no observable variation in secondary structure. It seemed that the proteins changed their structures from higher order to lower order under some denaturalized conditions.

The catalytic cofactor FAD is located between four-helix bundles in the helical domain in CPD photolyases [13]. The abrupt increase in the fluorescence intensity (Fig. 4b) after 170 $\mu W/cm^2$ UV-C irradiation, suggests that the helix bundle was loosened and FAD probably left the natural nonpolar environment [14] into the outside solutions, though CD spectra assay (Fig. 3) indicates that the secondary structure such as α-helix and β-sheet changed only slightly under the same conditions. Since the substrate binding depends on the hole formed by the four-helix bundles [14], the invariable binding activity suggests that the helix bundles loosened not too much after varying doses of UV-C irradiation (Fig. 5a).

The lost catalytic activity indicates that the Pyr dimers could not connect the FAD cofactor directly in UV-damaged photolyase, due to the FAD deviating from its natural site. As a result, the DNA lesions could not be photorepaired and the photolyase could not dissociate from the DNA (Fig. 5b).

In vivo experiment indicates that the photolyase can photorepair DNA lesions efficiently after UV-C flux of 25.5 $\mu W/cm^2$, but it lost the catalytic activity under the same UV flux *in vitro*. This is probably because the *in vivo* environment is more suitable for the enzyme activity of the photolyase than that *in vitro*, and that the photolyase can be protected *in vivo* by functional molecules such as heat-shock proteins [15] or replaced by new synthesized proteins.

Photolyases are important and efficient in reversing UV-induced Pyr dimers to monomers. Since photolyase is protein in nature, it would be damaged by excess UV radiation. At UV-C fluxes $\geq$170 $\mu W/cm^2$, the damage to the tertiary structure of the photolyase is evident. When the photolyase was irradiated by UV-C at 1700 $\mu W/cm^2$ for 5 min, it could be easily seen by eye that the photolyase was aggregated (data not shown).

In this work, we have investigated the influence of UV-C on photoreactivation by *Synechococcus* sp. PCC 7942 photolyase *in vivo* and *in vitro*, and analyzed the structural changes after varying doses of UV-C irradiation. Our results indicate that photoreactivation plays an important role in repairing UV-induced DNA lesions under UV-C fluxes $\leq$25.5 $\mu W/cm^2$, but it will be inactivated by severe UV-C radiation due to structural changes. To date, there is little information on the value of UV-C flux, especially that during the origin of life. These results might be helpful to evaluate the UV-C flux at that time.

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