Isolation of a UV Endonuclease from the Cyanobacterium *Synechocystis* PCC 6308

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(Received 13 October 1981; revised 4 January 1982)

A UV endonuclease with activity towards UV-irradiated lambda DNA but no activity towards non-irradiated DNA was isolated from a crude lysate of the unicellular cyanobacterium *Synechocystis* PCC 6308. The enzyme has a molecular weight between 15000 and 18000. ATP was essential for maximum enzyme activity and the optimum pH was 7.5–7.8.

INTRODUCTION

A variety of mechanisms have been shown to be present in eukaryotic and bacterial cells for the repair of UV-damaged DNA. These include photoreactivation, excision repair, post-replication and 'SOS' repair (reviewed by Moseley & Williams, 1978; Hanawalt *et al.*, 1979). In the cyanobacteria, considerable evidence has accumulated for the photoreactivation of UV damage (reviewed by Van Baalen, 1973; Delaney *et al.*, 1976; Ladha & Kumar, 1978) and partially purified photoreactivating enzyme has been isolated and characterized (Saito & Werbin, 1970). Williams *et al.* (1979) reported the presence of a very efficient photoreactivating system in *Synechocystis* PCC 6308 (*Gloeocapsa alpicola*). The evidence for dark repair of UV damage in the cyanobacteria has, however, been much less conclusive (reviewed by Van Baalen, 1973; Delaney *et al.*, 1976; Ladha & Kumar, 1978). Recently, Williams *et al.* (1979) presented preliminary evidence for excision repair in *Synechocystis* based on the loss of photoreversibility during dark liquid holding of irradiated cells due to competition between the photoreactivating system and the dark repair system. Geoghegan (1980) further confirmed the presence of an excision repair system by the use of inhibitors of excision repair. When caffeine, coumarin or pyronin Y were incorporated into the dark liquid holding medium, a delay in the onset of loss of photoreversibility was observed due to the inhibition of excision repair functions. In this communication direct evidence of cyanobacterial excision repair is presented by the isolation, from the unicellular cyanobacterium *Synechocystis*, of the enzyme involved in the first step of the pathway: the UV endonuclease.

METHODS

Strains. *Gloeocapsa alpicola* (Lyngbye) Bornet, obtained from the Culture Centre for Algae and Protozoa, Cambridge, England (Cat. no. 1430/1), has been reassigned to the genus *Synechocystis* and is specified by the Pasteur Culture Collection no. PCC 6308 (Rippka *et al.*, 1979). *Escherichia coli* CSH45, F⁻ *lac thy trpR* lysogenic for phage lambda (*cI857 S7*), obtained from the Department of Genetics, Trinity College, Dublin, was used as a source of phage lambda DNA. The mutation in the phage *cI* gene produced a heat labile repressor and an additional mutation (*S7*) prevented cell lysis.

Culture conditions. Liquid cultures of *Synechocystis* were grown in a modified medium of Allen (1968), as described by Williams *et al.* (1979). Solid growth medium was prepared by adding 3.6% (w/v) agar (Difco) to an equal volume of double-strength liquid medium. Cultures were incubated at 30 ± 1 °C at approximately 50% relative humidity under constant illumination with white fluorescent light at 2000 lx. Liquid cultures were orbitally shaken at 120 rev. min⁻¹. Liquid cultures of *E. coli* were prepared by inoculating 200 ml Luria broth (Miller, 1972)

with 20 ml overnight cultures of *E. coli* grown in the same medium at 30 °C. After incubating the cultures for 30 min at 30 °C, they were transferred to a water-bath at 42 °C and shaken vigorously for 20 min to induce prophage. Following phage induction, the cultures were incubated at 37 °C for a further 3 h after which they were harvested by centrifugation at 5000 g for 20 min.

Isolation of lambda phage DNA. A suspension of *E. coli* CSH45 in 10 ml buffer containing Tris/HCl pH 7.5 (0.02 M), NaCl (0.1 M), MgSO₄ (10⁻³ M) and gelatin (0.01%, w/v) was lysed by adding 1 ml chloroform and shaking the suspension for 10 min at 37 °C. Bovine pancreatic DNAase (10 µl) (Sigma) at a concentration of 1 mg ml⁻¹ in water was added and shaken slowly for 10 min at 37 °C. The DNAase treatment was repeated and the suspension was centrifuged at 12000 g for 15 min. The supernatant was then layered on to the top of a block CsCl gradient containing 10 ml CsCl with specific density of 1.6, 7.5 ml CsCl of specific density 1.5, and 7.5 ml CsCl of specific density 1.4. The gradients were centrifuged at 37000 g for 3 h in an FA 60 Ti head of a Beckman L5-65B ultracentrifuge. Following centrifugation, a phage band with a yield of 10⁸ phage ml⁻¹ was removed from the gradient. DNA was extracted from the phage solution by combining 1 ml phenol saturated with 0.1 M-Tris/HCl pH 7.5 with 1 ml phage solution and allowing the two solutions to mix on a roller for 30 min at room temperature. The mixed solutions were then cooled in an ice-bath and centrifuged at 600 g for 5 min to separate the two layers. The aqueous layer was dialysed four times against 100 ml DNA buffer (Geoghegan, 1980) at 4 °C, each dialysis taking 2 h, and stored at -20 °C until required. ³H-labelled phage lambda DNA was isolated from *E. coli* CSH45 *thy* cultures incubated in the presence of [³H]thymidine with a specific activity of 57.7 mCi mmol⁻¹ (2.13 GBq mmol⁻¹). Irradiated lambda DNA was obtained by exposure to 3.5 × 10⁴ µJ mm⁻² UV (253.7 nm) from a mercury discharge lamp (Griffin and George). All operations were carried out under yellow light (550–700 nm) to prevent photoreactivation.

Purification of UV endonuclease. Samples (25 ml) of frozen (-20 °C) cyanobacterial suspension (4 × 10⁹ cells ml⁻¹) in 0.01 M-Tris/HCl (pH 7.5) were passed three times through the orifice of an X-Press pre-cooled to -20 °C. The final lysate was stored at -20 °C. Cell debris was removed by centrifugation at 30000 g for 30 min and the supernatant was adjusted to 300 ml using Tris/HCl pH 7.5 (fraction 1). (NH₄)₂SO₄ was added to 70% saturation. The solution was stirred for 1 h and centrifuged (30000 g, 30 min). (NH₄)₂SO₄ was added to the supernatant to a concentration of 90% (w/v), stirred for 1 h and again centrifuged. The precipitate was dissolved in 200 ml 5 mM-potassium phosphate buffer, pH 7.5 (fraction 2) and applied to a DE-52 cellulose column (40 × 2.5 cm) pre-equilibrated with buffer, at 0.5 ml min⁻¹. The column was eluted with the phosphate buffer and 9 ml fractions were collected and assayed for UV-specific endonuclease, and for protein using the Lowry method. When no activity was found in the fractions, a gradient (350 ml) from 5 mM–0.2 M-potassium phosphate buffer (pH 7.5) was applied to the column; 9 ml fractions were collected and assayed for protein and endonuclease activity. The endonuclease fractions were pooled (fraction 3) and the pH adjusted to 6.5 before being applied to a hydroxylapatite column (20 × 1 cm). After the column was washed with 50 ml 0.01 M-potassium phosphate buffer (pH 6.5), a linear gradient of this buffer and 0.3 M-potassium phosphate buffer (pH 6.5) was applied. Fractions (5 ml) were collected and assayed for protein and UV endonuclease activity and the active fractions pooled (fraction 4).

Measurement of UV endonuclease activity. UV endonuclease activity was assayed by measuring the conversion of irradiated high molecular weight phage lambda DNA to low molecular weight fragments (Geoghegan, 1980), using non-irradiated DNA as a control. Activity was expressed as the ratio of the heights of the peaks of non-irradiated and irradiated DNA at A₂₆₀. The reaction mixture contained, in a final volume of 450 µl: DNA (2.5 µg in 50 µl), 1 mM-MgCl₂ (125 µl), 50 mM-Tris/HCl pH 8.0 (125 µl), 18 mM-ATP (50 µl) and cell lysate or enzyme fraction (100 µl). Throughout the purification, the protein concentration used in the enzyme assay varied from 7.55 mg ml⁻¹ (fraction 1) to 8.0 µg ml⁻¹ (fraction 4). Mg²⁺ ions were included in the reaction mixture since the UV endonucleases of *E. coli* and *Micrococcus luteus* have been shown to be stimulated by, but not dependent upon Mg²⁺. Assays were routinely carried out for 15 min at 37 °C under yellow, non-photoreactivating light (550–700 nm). Reactions were started by adding the lysate or enzyme containing fraction and stopped by adding NaOH (final concentration 0.1 M) followed by neutralization with HCl. For the analysis of the reaction products, 100 µl samples of each reaction mixture were applied to Sepharose-4B columns (8 × 1 cm) and eluted with 2 mM-Tris/HCl (pH 7.5) plus 1 mM-MgCl₂. The eluate was monitored at 260 nm in the flow cell of an LKB Uvicord Spectrophotometer. The optimum time required for the endonuclease reaction was determined with fraction 4, using varying reaction times from 5 to 30 min with the standard reaction mixture. The optimum pH for endonuclease activity was determined using fraction 4 and the standard reaction procedure was followed using an incubation time of 15 min and varying pH values from 5 to 9. The requirement of ATP for enzyme activity was determined at the start of the purification procedure by incorporating 2 mM-ATP into the reaction mixture using fraction 1 as the enzyme source. The effect of caffeine on endonuclease activity was also investigated by incorporation of 5 × 10⁻² M-caffeine into the reaction mixture using fraction 4 as the enzyme source. To determine the effect of protein concentration on endonuclease activity, ³H-labelled lambda DNA was used with varying amounts of fraction 4 giving a final protein concentration from 0 to 2.9 µg ml⁻¹. The reactions were carried out under standard conditions. Samples (100 µl) of the reaction mixture were layered on to 5–20% sucrose linear

Fig. 1. Typical elution profile of irradiated (●), and non-irradiated (○) lambda DNA on a Sepharose-4B column, after treatment with UV endonuclease.

gradients and centrifuged (200000 *g*, 120 min). Fractions (9 drops) were collected directly into vials of scintillation mixture and counted in a Tri-Carb liquid scintillation spectrometer (Geoghegan, 1980). Non-irradiated DNA was used as a control. The activity was expressed as a percentage of the total radioactivity in the undegraded state (Geoghegan, 1980).

Molecular weight estimation. The molecular weight of the UV endonuclease was estimated by applying 100 μ l of fraction 4 to a Sephadex G-75 column, using the elution volumes of dextran blue, ovalbumin, trypsin, lysozyme, RNAase and insulin as references.

RESULTS

A UV-specific endonuclease with activity towards UV-irradiated lambda DNA, but not non-irradiated DNA, was isolated and purified from *Synechocystis* PCC 6308. Following each step in the purification procedure, the protein content and the endonuclease activity, measured by the Sepharose-4B assay and expressed as the ratio of the heights of the peaks at A_{260} of irradiated and non-irradiated DNA, was estimated for each fraction. Figure 1 illustrates the typical profile of endonuclease activity on both irradiated and non-irradiated DNA following separation of the reaction products on a Sepharose-4B column. The major difference in the two elution patterns is the introduction of low molecular weight irradiated DNA (9–14 ml of eluate) following incubation with the enzyme (fractions 1–4) and the absence of this low molecular weight shoulder with non-irradiated DNA. The enzyme activity was calculated by comparing the heights of the DNA peaks at the 7 ml point of the elution. This general representation of enzyme activity is suitable for enzyme isolation purposes, provided that the UV dose for each substrate DNA is constant.

The UV endonuclease in the crude lysate (fraction 1; total protein 7.55 mg ml⁻¹, enzyme activity 1.15:1) was partially purified by (NH₄)₂SO₄ precipitation (fraction 2; total protein 2.3 mg ml⁻¹, enzyme activity 1.22:1). It was further purified by elution from a DE-52 cellulose column in approximately 100 ml in the 0.13 M-phosphate area of the gradient (fraction 3; total protein 1.0 mg ml⁻¹, enzyme activity 1.35:1). The greatest purification of the UV endonuclease was effected in the final step of the procedure when fraction 3 was separated on a hydroxylapatite column. The UV endonuclease eluted in approximately 50 ml in the 0.28 M-phosphate area (fraction 4; total protein 8 μ g ml⁻¹, enzyme activity 1.6:1).

The purified enzyme was stable when frozen, but maximum stability was only maintained for 1 week when stored at 4 °C. The molecular weight of the enzyme was determined to be between 15000 and 18000. The UV endonuclease was active at pH 5.5–9.0, having maximum activity at pH 7.5–7.8. The protein concentration giving the highest activity was

2.3–2.5 $\mu\text{g ml}^{-1}$, using fraction 4 as a source of enzyme. The optimum time for the *Synechocystis* endonuclease reaction to go to completion using 2.5 μg lambda DNA irradiated at $3.5 \times 10^4 \mu\text{J mm}^{-2}$ was 12–15 min.

The endonuclease reaction was not dependent on Mg^{2+} since no variation in enzyme activity occurred when the enzyme assay was carried out either with or without EDTA (10^{-2} M). However, MgCl_2 was included in all endonuclease reactions since it is thought to stimulate the *E. coli* UV endonuclease. The enzyme was dependent on ATP (2 mM) for maximum activity since there was a 24% reduction in the difference between the irradiated and non-irradiated DNA peaks eluting from the Sepharose column when ATP was excluded from the reaction, thereby reducing the enzyme activity from 1.15:1 to 1.06:1. When caffeine (5×10^{-2} M) was included in the reaction mixture, the difference in the heights of the DNA peaks eluting from the Sepharose column was reduced by 60% causing a decrease in enzyme activity from 1.6:1 to 1.18:1.

DISCUSSION

The exact reaction conditions were important when carrying out UV endonuclease analysis. Since crude extracts were being used as a source of endonuclease, it was possible that these extracts could contain photoreactivating enzyme which would also have activity towards the dimer substrate. Photoreactivating wavelengths of light were therefore rigorously excluded from all reaction conditions.

ATP was necessary for maximum enzyme action. Since nucleolytic reactions are considered to be endothermic (Seeberg & Strike, 1976) there is no obvious requirement for the energy of ATP in the incision reaction. The ATP may serve to activate or maintain the association of the UV-induced dimer and UV endonuclease complex.

The *in vitro* incorporation of caffeine into the endonuclease reaction decreased the activity of the enzyme by 60%. Caffeine induces a delay in the onset of loss of photoreversibility during *in vivo* dark liquid holding (Williams *et al.*, 1979; Geoghegan, 1980) and it has been proposed that this is indirect evidence for a dark repair system in *Synechocystis*. The demonstration of the inhibitory effect of caffeine on the UV endonuclease *in vitro* substantiates this claim. It is now evident that *Synechocystis* contains an excision repair system for UV damage which is comparable to the repair system of other bacteria. This is the first report of the isolation of a UV endonuclease from a cyanobacterium and the first direct demonstration of an excision repair system in this group of organisms.

This work was financed in part by Euratom under its Biology-Health Protection Programme, Contract no. 127174-1 BIO EIR.

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