Identification and amplification of the *E. coli* *phr* gene product

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ABSTRACT

We have constructed a series of multicopy plasmids that complement mutations in the *phr* gene of *Escherichia coli*. By subcloning into a *tac* plasmid vector we obtained a *phr* plasmid that upon induction overproduces two proteins of Mr's 49,000 and 20,000. Tn1000 insertions into the *phr* gene caused the disappearance of the 49,000 dalton protein, thus demonstrating this protein to be the *phr* gene product, DNA photolyase. The photolyase encoded by the *phr* gene makes up about 15% of total cellular proteins after induction of cells carrying a *tac-phr* plasmid. This protein binds specifically to UV (254 nm) irradiated DNA and upon exposure to near UV (300-500 nm) illumination repairs the UV damage and dissociates from DNA.

INTRODUCTION

Exposure of *E. coli* cells to near UV light (300-500 nm) after irradiation with far UV (200-300 nm) reverses some of the effects of far UV radiation such as growth delay, mutagenesis and death. This phenomenon is called photoreactivation and is mediated by the enzyme deoxyribodipyrimidine photolyase (EC.4.-1.99.3., DNA photolyase, photoreactivating enzyme, PRE). Photolyase accomplishes its function by catalyzing photomonomerization of pyrimidine dimers produced in DNA by far UV radiation. The enzyme acts on DNA by a two-step reaction mechanism: 1) it binds to dimer-containing DNA (UV-DNA) in a light-independent step to form a stable enzyme-substrate complex and 2) upon exposure to near UV breaks the two bonds of the cyclobutone ring between the two pyrimidines of the dimer and dissociates from DNA (1).

In *E. coli* K-12 there are only about 10 molecules of photolyase per cell (2), a fact which makes the purification of the enzyme exceedingly difficult. To overcome this difficulty several groups have attempted to overproduce the

enzyme by genetic means. Sutherland *et al.* (3,4) mapped the gene for photolyase between *gal* and *attλ* on the standard *E. coli* genetic map and obtained a λ dgai phage which upon induction of lysogens led to overproduction of a 35,000 dalton protein having photolyase activity. However, Sancar and Rupert (5,6) and Youngs and Smith (7) found that all Δ (*gal-attλ*) mutants tested were photoreactivable and mapped the photoreactivation gene, *phr*, at 15.7 min (8). Inactivation of this gene by mutation causes a complete loss of photoreactivability in the mutant strain, *in vivo* (9) and *in vitro* (10). We previously reported (11) cloning of *phr* on the multicopy plasmid pMB9 and demonstrated that the cloned gene led to overproduction of photolyase by gene dosage effect. In this paper we describe the construction of a *tac-phr* plasmid which upon induction produces a protein of Mr 49,000 to a level of about 15% of total cellular proteins. The amplified protein binds to UV-DNA in the dark and upon illumination repairs the DNA and dissociates from it.

MATERIALS AND METHODS

Bacterial strains and plasmids.

All strains used are *E. coli* K-12 derivatives. CSR603 (*recA1 uvrA6 phr-1*) was obtained from the *E. coli* Genetic Stock Center, Yale University. RB3 is a derivative of W3110 which carries *Flaci*^Q. It was obtained from Dr. R. Brent of Harvard University. CSR603/*Flaci*^Q was constructed by mating RB3 with CSR603 and selecting for Pro⁺, streptomycin resistant exconjugants. The *tac* plasmid p*tac12* is a pBR322 derivative that carries a hybrid *trp*(-35)-*lac*(-10) promoter on a 280 bp EcoRI-PvuII fragment. The plasmid was constructed by E. Amann and J. Brosius of Harvard University and provided to us by Dr. Amann. The *tac* promoter fragment also carries the *lac* repressor and therefore is repressed by *lac* repressor and induced by the gratuitous *lac* inducer isopropyl-thio-D-galactoside (IPTG). By inserting the *tac* fragment of this plasmid into the EcoRI-PvuII sites of pBR328 we obtained pUNC09 which was used to construct plasmids which amplify photolyase. The *phr* plasmid pCSR604 has been described previously (11). The constructions of pCSR606, pMS2, pMS725 and pMS969 are described in the Results section.

Construction of *phr::Tn1000* plasmids.

The Tn1000 ($\gamma\delta$) transposon of F factor was inserted into *phr* carried by the photolyase overproducing plasmid pMS725 by a modification of the methods of Guyer (12) and Sancar and Rupp (13,14) as follows: RB3/pMS725 (*Flaci*^QPhr⁺TetR Str^S) was mated with CSR603 (F⁻Phr⁻Tet^SStr^R) and the mating mixture was plated onto Luria plates containing tetracycline (20 μ g/ml) and streptomycin (100

µg/ml). After incubating the plates at 37°C overnight single colonies were picked and grown in selective medium to stationary phase. The cultures were then diluted 1/10 and 5 µl samples were spotted onto Luria plates (50 culture per plate). The plates were irradiated with 0.5 J/m² of UV, then photoreactivated for 1 hr and incubated at 37°C. Patches of Phr⁺ cells grew confluent on these plates whereas patches of Phr⁻ cells contained a few colonies or none at all. These latter isolates were presumed to carry pMS725 derivatives that had Tn1000 insertion in the *phr* gene. The reason for using *Flaci*^Q instead of F as the source of Tn1000 in this experiments is the observation that *tac* plasmids are unstable in strains without *laci*^Q (unpublished data); by using *Flaci*^Q as the donor of Tn1000 we insure that every cell that receives the *phr-tac* plasmid pMS725 also receives *laci*^Q carried by *Flaci*^Q.

UV irradiation and photoreactivation.

General Electric germicidal lamp G8T5 emitting mainly at 254 nm was the UV source. The fluence rate was measured with a UVX Digital Radiometer from Ultraviolet Products Inc. GE Black Light F15T8/BLB lamp covered with Saran wrap (to filter out radiation below 300 nm) was used for photoreactivation. The photoreactivated samples were placed at approximately 40 cm from the lamp and unless otherwise stated photoreactivating light exposure was for 1 hr. Experiments involving photoreactivation were done under GE "Gold" fluorescent light.

Purification of photolyase.

The enzyme was prepared from the photolyase overproducing strain CSR603/*Flaci*^Q/pMS969 by sequential chromatography on phenyl sepharose, hydroxylapatite and DNA-cellulose. The details of the purification procedure will be published elsewhere.

Photolyase assay.

The enzyme was assayed *in vitro* by adapting the classical *Haemophilus* transformation assay (15) to *E. coli*. Briefly the assay is as follows. The reaction mixture contained in 50 µl: Tris·HCl 50 mM pH 7.2/NaCl 10 mM/EDTA 1 mM/DTT 10 mM/UV-pBR322 (30 J/m² UV) 0.5 µg/photolyase 0-10 ng. The mixture was illuminated at 20°C for 1 hr then added to 200 µl competent CSR603 cells; transformation was carried out by the standard method. After outgrowth for 2 hrs samples were plated on Luria plates containing tetracycline at 20 µg/ml. The difference between the number of transformants obtained with illuminated sample and dark-kept controls is a measure of photolyase activity.

Other methods.

Restriction nucleases, Bal31 nuclease, T4 DNA ligase and IPTG were obtained from Bethesda Research Laboratories and were used as recommended by the sup-

plier. Plasmid minipreparations, agarose and SDS-polyacrylamide gel electrophoreses were done according to established methods. SDS-polyacrylamide gels were scanned with a Fiber Optic Scanner Model 800 (Kontes Scientific Instruments) and the peaks were integrated with a Model 3390A Integrator from Hewlett Packard. Cell free extracts were prepared by sonication of cell suspensions (in Tris 50 mM pH 8.0/EDTA 1 mM/ β -mercaptoethanol 10 mM/sucrose 10%) for 60 sec with a Bronson Model W185 cell sonifier set at maximum output, followed by removal of cell debris by centrifugation at 44,000 rpm for 1 hr. in a Beckman Ti70 rotor.

RESULTS

Subcloning of *phr* gene.

The original *phr* plasmid we isolated, pCSR604, contained two chromosomal EcoRI fragments 18 and 10 kbp in size inserted into the pMB9 vector (11). The *phr* gene was subcloned from this plasmid by inserting a 6kbp BamHI-EcoRI* fragment of pCSR604 into the BamHI-EcoRI sites of pBR322. The resulting plasmid was named pCSR606. By digesting pCSR606 with AccI and self-ligating we obtained pMS2 (Figure 1). This plasmid, like pCSR604 and pCSR606, fully complements the *phr-1* mutation (Figure 2) as well as deletion mutants of *phr* (data not shown). To put the *phr* gene under *tac* control we used the *tac* plasmid pUNC09 whose restriction map is shown in Figure 3. The 1.9 kbp AccI-PstI fragment of pMS2 was blunt-ended at the AccI site by treating with Bal31 and then inserted into the PvuII-PstI sites of pUNC09 to obtain pMS725. This plasmid also fully

Figure 1 Subcloning of the *phr* gene. pCSR606 was generated by inserting a BamHI-EcoRI* fragment of pCSR604 (not shown) into the BamHI-EcoRI sites of pBR322. By deleting the region between 2.1 and 7.9 kbp of pCSR606 by AccI digestion and self-ligation pMS2 was generated. (EcoRI) indicates EcoRI-EcoRI* junction. The locations and directions of transcription of β -lactamase (*bla*), photolyase (*phr*) and twenty kilodalton protein (*tkp*) genes are represented by arrows. Vector DNA is indicated by the double line.

Figure 2 Complementation of *phr-1* mutation by *phr* plasmids. To obtain these survival curves appropriate dilutions of stationary phase cultures were plated on Lauria plates which were irradiated with 254 nm at the indicated fluences and then either kept in the dark or photoreactivated for 1 hr at 22°C. Colonies were counted after incubation at 37°C overnight. The closed symbols are for dark survivals and the open symbols for survivals after photoreactivation.

▼△ CSR603   ▲△ CSR603/*FlacI*^Q
 ●○ CSR603/pCSR606   ▲△ CSR603/*FlacI*^Q/pMS725
 ■□ CSR603/pMS2   ◆◇ CSR603/*FlacI*^Q/pMS969

Figure 3 Construction of *tac-phr* plasmids. The *tac* promoter is indicated by the heavy arrow. Other symbols are the same as in Figure 1.

Figure 4 Amplification and identification of the *phr* gene product. The proteins encoded by cells carrying pMS725 and its Tn1000 insertion derivatives were separated on an SDS-polyacrylamide gel which was stained with Coomassie blue and photographed. Each lane contains 150 μ l culture of IPTG-induced CSR603/*Flac*^Q containing the following plasmids: Lane 1, none; Lane 2, pMS725; Lanes 3 through 6, pMS725::Tn1000 #2, 7, 15 and 17 respectively; Lane 7, Mr markers: phosphorylase b (93,000), bovine serum albumin (67,000), ovalbumin (45,000) and carbonic anhydrase (29,000). Lanes 8 through 14, pMS725::Tn1000 #19, 23, 28, 32, 35, 40 and 47 respectively. The (+) and (-) signs indicate whether the cells are *Phr*⁺ or *Phr*⁻. Induction with IPTG (1 mM) was for 6 hrs.

complements the *phr-1* mutation of CSR603 (Figure 2).

Overproduction and identification of DNA photolyase.

To identify the *phr* gene product we analyzed on SDS-polyacrylamide gels the proteins made by CSR603/*Flac*^Q/pMS725 following induction with IPTG. We found that the induced cells greatly overproduced two proteins of Mr's 49,000 and 20,000 and to a lesser extent a third protein of Mr 29,000 (Figure 4). This last protein co-migrates with β -lactamase coded by pBR322 (data not shown) and therefore it was presumed to be β -lactamase of the vector. To find out which of the remaining two proteins was photolyase we isolated *phr*::Tn1000 derivatives of pMS725 and analyzed their protein patterns following induction with IPTG. As is seen in Figure 4, insertion of Tn1000 into *phr* resulted in disappearance of the Mr 49,000 protein which was therefore identified as the *phr* gene product. These results also show that the directions of transcription of *phr* and of *tkp* (for twenty kilodalton proteins) are the same

Figure 5 Mapping of the *phr* gene. The sites of Tn1000 insertions that inactivate *phr* are indicated by arrows perpendicular to the restriction map of pMS725. Centrifugal arrows are for Tn1000 insertions in $\gamma\delta$ -clockwise orientation and the centripetal arrows for insertions in the opposite orientation.

Figure 6 Amplification of photolyase. A. Synthesis of photolyase in uninduced (Lane 1) and IPTG (1 mM)-induced (Lane 2) CSR603/*FlacI*⁰/pMS969. Induction was for 6 hrs. PRE=photolyase, BLA=β-lactamase. This is a photograph of a Coomassie blue-stained SDS-polyacrylamide gel. B. Densitometric scan of Lanes 1 and 2 of the gel shown in A. The peaks corresponding to photolyase and β-lactamase are indicated. Integration of the areas under the peaks indicates that photolyase makes up 15.76% of total cellular proteins in induced cells.

as that of *tac* on pM5725 as shown in Figure 5. The *tkp* gene was assigned to the 5' side of *phr* as this is the only region of chromosomal DNA not occupied by *phr* and of sufficient length to encode a Mr 20,000 protein. This assignment was confirmed during analysis of a second *tac-phr* plasmid, pMS969. The construction of this plasmid was similar to that of pMS725 except that Bal31 digestion was allowed to proceed for an average of 500 bp from the AccI site in pMS2. As is seen in Figures 2 and 3, pMS969 is *phr*⁺ and contains only about 1.4 kbp of chromosomal DNA which is the length predicted for a protein of Mr 49,000. When cells carrying this plasmid are induced with IPTG, the Mr 20,000 protein is absent while photolyase is made in large quantities, constituting about 15% of total cellular proteins after 6 hrs of induction (Figure 6). This corresponds to about a 15,000-fold amplification of the enzyme.

Binding of photolyase to UV-DNA.

One of the unique properties of photolyases is binding to UV irradiated DNA in the dark and dissociating from it upon exposure to near-UV (photoreactivating) light (1). This binding stabilizes the yeast enzyme against heat inactivation (1), an observation that was recently exploited in purifying that enzyme (16). To demonstrate that the *phr* gene product has this property we did the following experiment: a cell-free extract of induced CSR603/*Flac*^Q/pMS969 was mixed with either non-irradiated DNA or increasing concentrations of UV-DNA; the mixture was heated at 69°C for 150 sec, cooled on ice, then centrifuged to remove the denatured proteins. The supernatant was analyzed on an SDS-polyacrylamide gel (Figure 7). Heating in the absence of DNA or in the presence of non-irradiated DNA at a concentration of 200 µg/ml results in denaturation and precipitation of photolyase, whereas in the presence of increasing concentrations of UV-DNA increasing amounts of photolyase are protected against heat denaturation and stay in the supernatant. If the UV-DNA-photolyase mixture is exposed to photoreactivating light prior to the heating step the repaired DNA no longer protects photolyase against heat denaturation and the enzyme precipitates. Therefore we conclude that the 49,000 dalton protein is a true photolyase.

All photolyases studied to date (17,18) have co-factors. If this is also true of *E. coli* photolyase then the preceding experiment indicates that in induced cells both apo-enzyme and co-factor are present in roughly similar amounts; limiting amount of co-factor necessary for the second step (photolysis) of the enzymic reaction would result in failure of photolyase bound to irradiated DNA to be released upon photoexposure.

Biological activity of DNA photolyase.

The ultimate test for identification of a protein as a DNA photolyase is the

Figure 7 Protection of photolyase from heat denaturation by UV-DNA. This is a photograph of a SDS-polyacrylamide gel that was stained with Coomassie blue. Lane 1, total cellular proteins from CSR603/*FlacI^S*/pMS969. Lane 2, total cellular proteins of the same strain after induction with IPTG for 6 hrs. Lane 3, cell-free extract of induced cells. Lanes 4 through 8, supernatants of cell-free extracts that were heated in the presence or absence of calf-thymus DNA and then centrifuged. The DNA concentrations were as follows: Lane 4, none added; Lane 5, non-irradiated DNA 200 $\mu\text{g/ml}$; Lane 6 through 8, UV-DNA (10^5J/m^2) at 50, 100 and 200 $\mu\text{g/ml}$, respectively. Lane 9, the same as Lane 8 except the sample was photoreactivated before heating. Lanes 4 through 9 contained three times as much sample as Lane 3. Lane 10, purified photolyase (2.5 μg). Lane 11, Mr markers: bovine serum albumin (67,000), ovalbumin (45,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000) and trypsin inhibitor (20,000). α -amylase which reportedly has a Mr of 49,000 migrates anomalously on this gel system as a protein of Mr 59,000.

restoration by that protein of biological activity of UV-DNA in a light (300-500 nm) dependent reaction *in vitro*. To demonstrate such an activity for our enzyme, various amounts of the purified protein (Figure 7) were mixed with UV-pBR322, the mixture exposed to photoreactivating light and then used to transform competent *E. coli* cells to tetracycline resistance. Figure 8 shows that indeed the purified protein repairs pBR322 DNA in a light-dependent reaction and therefore is a true DNA photolyase.

Figure 8 Repair of UV-pBR322 by photolyase *in vitro*. The reaction mixtures contained the indicated amounts of photolyase and 0.5 μg of UV-pBR322 (300 J/m^2). ●, Non-photoreactivated; ○, photoreactivated for 1 hr. Non-irradiated pBR322 gave 3.1×10^4 transformants/ μg and UV-pBR322 gave 43 transformants/ μg under the same assay conditions.

DISCUSSION

In this paper we have described the identification and amplification of the *phr* gene product of *E. coli*. We have found that *phr* codes for a protein of Mr 49,000 which binds specifically to UV-DNA and repairs it in a light-dependent reaction. The major question that arises from this work is whether *E. coli* possesses two DNA photolyases because the protein we have identified as photolyase is clearly distinct from the Mr 35,000 protein having photolyase activity described by Sutherland and co-workers (3,4). Our data show that amplification of the *phr* gene product by itself leads to a parallel increase in photolyase activity, suggesting that if there are two structural photolyase genes these two genes do not code for subunits of the same enzyme. Similar amplification of the gene located at the *gal-att λ* interval reportedly results in increased photolyase activity reinforcing the idea that each gene product alone is capable of photoreactivating activity. Assuming that there are two independent photolyase genes one would expect that cells having mutations in either gene would still be *Phr*⁺. Indeed cells having $\Delta(\text{gal-att}\lambda)$ mutations are *Phr*⁺. A claim that these cells have a reduced rate of photoreactivation (19) has not been confirmed (7). On the other hand cells having a mutation in the *phr* gene are totally lacking photoreactivability *in vivo* (9) and *in vitro* (10). It is conceivable that *E. coli* produces a major photolyase encoded by the *phr* gene and a minor one which is encoded by a gene in the *gal-att λ* inter-

val and that the level or the activity of the latter is such that is not detectable in wild-type cells but it becomes measurable when greatly amplified by genetic means. Further work is needed to verify this hypothesis.

The second question that arises from this work is whether the photolyase encoded by the *phr* gene has a specific co-factor. Since photolyases mediate DNA repair only in the presence of near UV radiation it has been long suggested that they may have a chromophoric co-factor that absorbs light in this radiation range. Indeed such chromophores have been recently found in *Saccharomyces cerevisiae* (17) and *Streptomyces griseus* (18) enzymes and have been identified as flavins. On the other hand the *E. coli* enzyme isolated by Sutherland and co-workers seems to lack such a chromophoric co-factor; the enzyme has an absorption peak at around 260 nm and no significant absorption beyond 300 nm. The absorption peak at 260 nm was ascribed to an RNA co-factor and data was presented suggesting that the chromophore is formed during enzyme-substrate complex formation (20). The work reported in this paper does not directly address the question of whether or not our enzyme has a co-factor. However, the observation that several thousand-fold increase in the *phr* gene product, the Mr 49,000 protein, is accompanied by a similar increase in enzymatic activity strongly suggests that if there is a co-factor it must be an abundant molecular species to saturate the greatly elevated apo-enzyme levels. Flavins which act as co-factors for many enzymes in *E. coli* fit this description.

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