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# Purification of *Escherichia coli* DNA Photolyase*

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***Escherichia coli* photolyase is a DNA repair enzyme which monomerizes pyrimidine dimers, the major UV photoproducts in DNA, to pyrimidines in a light-dependent reaction. We recently described the construction of a *tac-phr* plasmid that greatly overproduces the enzyme (Sancar, G. B., Smith, F. W., and Sancar, A. (1983) *Nucleic Acids Res.* 11, 6667–6678). Using a strain carrying the overproducing plasmid as the starting material, we have developed a purification procedure that yields several milligrams of apparently homogeneous enzyme. The purified protein is a single polypeptide that has an apparent $M_r$ of 49,000 under both denaturing and nondenaturing conditions. The enzyme has no requirement for divalent cations and it restores the biological activity of irradiated DNA only in the presence of photoreactivating light. The purified photolyase has a turnover number of 2.4 dimers/molecule/min; this value agrees well with the *in vivo* rate of photoreactivation in *E. coli*.**

Cyclobutadipyrimidines or pyrimidine dimers are the major DNA photoproducts produced by ultraviolet (200–300 nm) radiation. If left unrepaired pyrimidine dimers can cause mutation, cancer, and death (1). In *Escherichia coli* there are several molecular mechanisms to eliminate pyrimidine dimers from DNA. Of these repair mechanisms, photoreactivation is of special interest because of its dependence on light. Photoreactivation is mediated by DNA photolyase, a flavoprotein (2) which binds to DNA-containing pyrimidine dimers in a light-independent step and converts the dimers to pyrimidines upon exposure of the complex to near-UV (300–500 nm) light. For a detailed study of the action mechanism of the enzyme, large quantities of photolyase are needed. We previously reported the construction of a photolyase-overproducing plasmid by joining a *tac* promoter to the photolyase apoenzyme gene, *phr*. We achieved an amplification level where photolyase constituted 15% of total cellular proteins (3). In this paper we describe a method for purifying milligram quantities of photolyase from these overproducing cells.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Plasmids*—*E. coli* CSR603 (*recA1 uvrA6 phr1*) was obtained from the *E. coli* Genetic Stock Center, Yale University. MS09 is CSR603 carrying $Flaci^Q$ and the *tac-phr* plasmid pMS969 ($Tet^R Amp^R Phr^+$) (Fig. 1). In our previous publications we referred to this strain as CSR603/$Flaci^Q$/pMS969 (2, 3). Cultures were routinely grown in Luria broth which in the case of MS09 contained tetracycline at 20 μg/ml. To induce photolyase, MS09 was grown to $A_{600}$ = 1.0 at which time isopropylthio-β-D-galactoside was added to 2 mM and incubation was continued for 12 h.

*Chemicals*—Column materials were obtained from the following sources: phenyl agarose from Pharmacia; hydroxylapatite (Bio-Gel HT), DEAE-agarose, and Bio-Gel ACA44 from Bio-Rad; single strand DNA-cellulose from P-L Biochemicals. Ethylene glycol was purchased from Fisher and isopropylthio-β-D-galactoside from Bethesda Research Laboratories.

*Buffers*—The cell lysis buffer contained 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol. Buffer A contained 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol. Buffer B is Buffer A containing 20% (v/v) gycerol. Buffer C contained 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 10 mM β-mercaptoethanol. Photolyase storage buffer contained 50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 50% glycerol (v/v).

*Methods*—Plasmid pBR322 was prepared from CSR603/pBR322 by sarkosyl lysis followed by equilibrium sedimentation in a CsCl-ethidium bromide density gradient. Protein concentration was determined by the method of Bradford (4) using Bio-Rad reagents. SDS[1]-polyacrylamide gels were prepared, run, and stained with Coomassie blue by established methods. The stained 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.

*Assay of DNA Photolyase*—A modified form of the classical transformation assay (5) was used to measure the increase in biological activity of UV-irradiated DNA as a result of photoreactivation *in vitro*. The substrate was UV-irradiated pBR322 and the host E. coli CSR603. This strain is totally deficient in repairing pyrimidine dimers, therefore a single pyrimidine dimer in incoming pBR322 DNA is sufficient to prevent transformation. The substrate was prepared by irradiating pBR322 with a UV fluence of 200 J/m$^2$ at a rate of 0.2 J/m$^2$/s and a DNA concentration of 100 μg/ml while magnetically stirring the DNA. This fluence resulted in 4.7 lethal photoproducts/

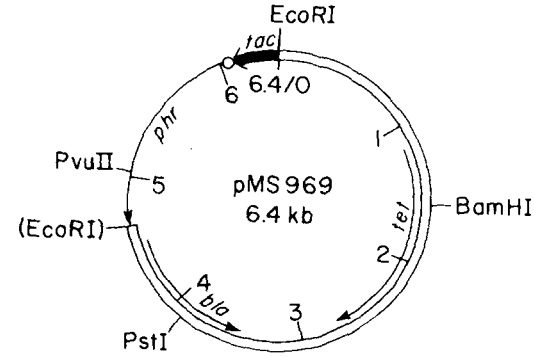


FIG. 1. **Restriction map of photolyase overproducing plasmid pMS969.** The *tac* promoter is indicated by the *heavy arrow*. The locations and directions of transcription of *phr*, *bla*, and *tet* genes are represented by *arrows* and the vector sequence by the *double line*. Restriction analysis indicates that the distance between the initiation codon of *phr* and the *tac* promoter fragment is 70 base pairs (data not shown).

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[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

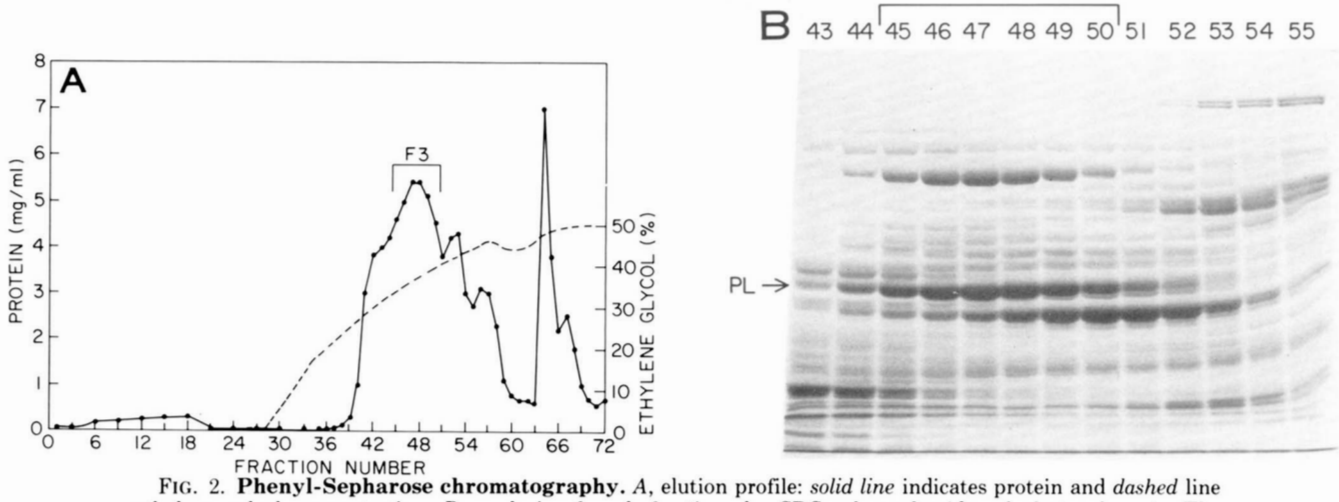


FIG. 2. **Phenyl-Sepharose chromatography.** *A*, elution profile: *solid line* indicates protein and *dashed* line ethylene gylcol concentration. *B*, analysis of peak fractions by SDS-polyacrylamide gel electrophoresis. The *numbers* indicate column fractions and *PL* the photolyase band. Twenty $\mu$l of each fraction were put onto the gel. The *bracketed fractions* were combined to obtain F3.

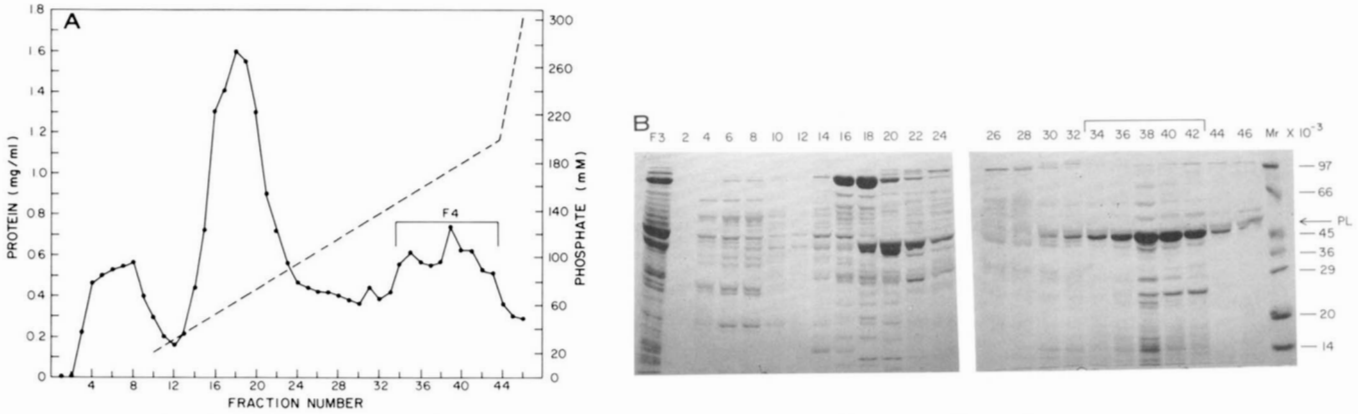


FIG. 3. **Hydroxylapatite chromatography.** *A*, protein elution profile: the *solid* and *dashed lines* indicate protein and phosphate concentrations, respectively. *B*, analysis of column fractions by SDS-polyacrylamide gel electrophoresis. *F3* is Fraction 3, the material that was loaded onto the hydroxylapatite column. The numbers of column fractions are indicated on *top* of each *lane*. The *last lane* contains $M_r$ standards. Twenty $\mu$l of each fraction were put onto the gel. The *bracketed fractions* were combined to obtain F4. *PL*, photolyase.

molecule as determined by measuring the survival of transforming efficiency (6). 90% of the lethal photoproducts were photoreactivable and were presumed to be pyrimidine dimers. The assay was conducted as follows. Substrate and enzyme were mixed in a 50-$\mu$l reaction buffer containing 50 mM Tris-HCl, pH 7.2, 10 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol; after incubation at 20 °C for 3 min the mixture was exposed at 20 °C to photoreactivating light (two G. E. Black Lights F15T8/BLB covered with Saran wrap to filter out radiation below 300 nm) at 20-cm distance. Samples were removed at intervals and added to competent CSR603 cells which were transformed according to standard procedures. After 2 h of outgrowth for expression of antibiotic resistance, triplicate samples were plated on Luria agar containing tetracycline at 20 $\mu$g/ml. The plates were incubated at 37 °C for 24 h and then counted. From the change in transformation efficiency as a result of photoreactivation, the amount of repaired pyrimidine dimers was calculated. One unit of photolyase is defined as the amount of enzyme that converts 1 pmol of pyrimidine dimers to pyrimidines in 1 h at 20 °C in accordance with the definition of Sutherland and Chamberlin (7). Unless otherwise stated, all experiments involving photoreactivation were done under yellow light from G. E. "Gold" flourescent lamps.

## RESULTS

### *Purification of Photolyase*

Upon induction, photolyase constitutes about 15% of total cellular proteins of *E. coli* MS09 (3) and therefore in SDS-polyacrylamide gels appears as the most prominent cellular protein. This enabled us to follow the purification of the enzyme by analyzing the various purification fractions on SDS-polyacrylamide gels. A functional assay was not conducted until the final purification step.

*Step I. Cell-free Extract—E. coli* MS09 was grown in 5 liters of Luria broth to $A_{600}$ = 1 and then induced with isopropylthio-$\beta$-D-galactoside for 12 h. From 5 liters of culture, 17 g of cells were obtained by centrifugation; the cells were resuspended in 100 ml of lysis buffer and frozen in a dry ice-ethanol bath and kept at −80 °C until further use. To lyse the cells the suspension was thawed at 0 °C overnight and the cells were sonicated with a Branson Model W185 sonifier set at maximum output for the small tip. Cell debris were removed by centrifugation at 32,000 × *g* for 20 min and then at 120,000 × *g* for 1 h to obtain a clear cell-free extract (Fraction 1, 100 ml).

*Step II. Ammonium Sulfate Precipitation*—43 g of ammonium sulfate were added to Fraction 1 over a period of 1 h with gentle magnetic stirring and the stirring was continued for another hour before collecting the precipitate by centrifugation. The precipitate was dissolved in Buffer A containing 10% saturated ammonium sulfate (Fraction 2, 130 ml).

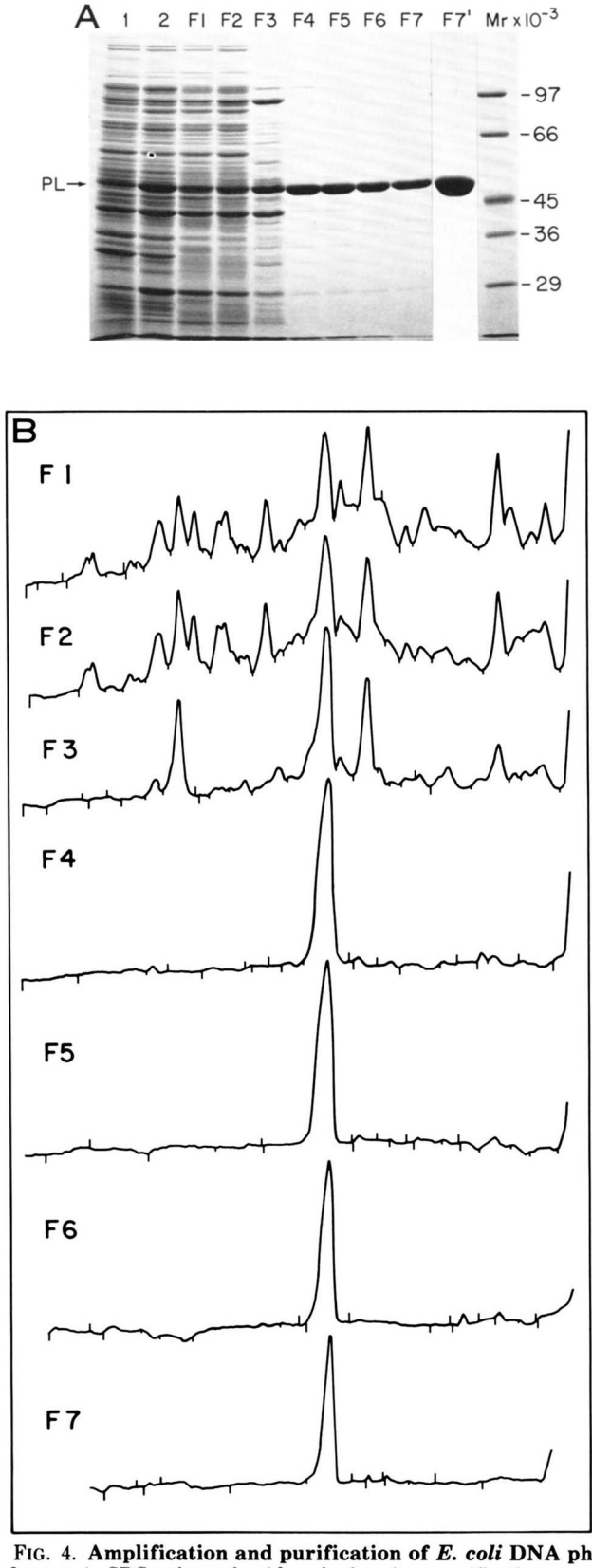


FIG. 4. **Amplification and purification of *E. coli* DNA photolyase.** *A*, SDS-polyacrylamide gel of various purification stages. *Lane 1*, uninduced MS09 cells; *Lane 2*, induced MS09 cells; *Lanes F1–F7*, samples of various purification stages. *F7'* contains a sample of fraction 7 from a different photolyase preparation. The samples contained 43, 52, 25, 10, 10, 10, 12.5, and 40 μg of protein, respectively. The *last line* contains $M_r$ standards. *PL*, photolyase. *B*, densitometric scan of *F1* through *F7 lanes* of the gel shown in *A*. By integrating the peaks in this scan the amount of photolyase in each fraction was determined. Bands containing less than 0.1 μg of protein were not measurable by the densitometer (data not shown) and as a result the last two purification fractions (*F6* and *F7*) which contained some minor bands were recorded as 100% pure photolyase. Assuming 5 contaminating bands at less than 0.1 μg each, we calculate that photolyase in fraction 7 is at least 95% pure. No contaminating bands were visable in *F7'*.

*Step III. Phenyl-Sepharose Chromatography*—Fraction 2 was loaded onto a 25-ml phenyl-Sepharose column equilibrated with 20% saturated ammonium sulfate in Buffer A. After loading, the column was washed with 50 ml of the same buffer and developed with a 250 ml gradient of 20% saturated ammonium sulfate in Buffer A to 50% ethylene glycol in Buffer A; fractions of 6.5 ml were collected. The fractions were analyzed for protein concentration by the Bradford assay and for protein composition by SDS-polyacrylamide gel electrophoresis. The results presented in Fig. 2, A and B, shows that photolyase is eluted at an ethylene glycol concentration of about 35%. The column fractions containing photolyase were combined and dialyzed against Buffer C containing 20% glycerol to obtain Fraction 3 (40 ml).

*Step IV. Hydroxylapatite Chromatography*—Fraction 3 was applied to a 20-ml hydroxylapatite column equilibrated with Buffer C; after washing with 25 ml of Buffer C containing 20% glycerol the column was developed with a phosphate gradient of 0.02 to 0.2 M, collecting 5-ml fractions. Then the column was washed with 25 ml of 0.4 M phosphate buffer. The protein elution and composition profiles of this column are shown in Fig. 3, *A* and *B*. As is apparent from the figure, photolyase eluted at around phosphate concentration of 0.18 M. The fractions containing the enzyme were combined and dialyzed against Buffer B containing 100 mM KCl (Fraction 4, 50 ml).

*Step V. DEAE-Agarose Chromatography*—Fraction 4 was applied to a 10-ml DEAE-agarose column equilibrated with Buffer A + 100 mM KCl. Photolyase did not bind to the column whereas some contaminating proteins did (Fig. 4). The wash-through of this column constituted Fraction 5 (50 ml).

*Step VI. DNA-cellulose Chromatography*—Fraction 5 was applied to a 7-ml single strand DNA-cellulose column equilibrated with Buffer B + 100 mM KCl and washed with 10 ml of the same buffer. The column was developed with a 50 ml gradient of 0.1 to 0.5 M KCl in Buffer B. Photolyase eluted at a KCl concentration of about 0.25 M (data not shown). The fractions containing the enzyme were combined to yield Fraction 6 (6 ml).

*Step VII. Phenyl-Sepharose Chromatography*—Fraction 6 was dialyzed against Buffer A + 20% saturated ammonium sulfate and loaded onto a 3-ml phenyl-Sepharose column equilibrated with the same buffer. Photolyase was eluted with a step gradient of ethylene glycol; the enzyme came off the column at an ethylene glycol concentration of 35–40%. Fractions containing photolyase were combined and dialyzed against photolyase storage buffer. The enzyme was frozen in a dry ice-ethanol bath and stored at −70 °C. The enzyme can be stored at this temperature for at least 3 months without any detectable loss of activity.

Analysis of the various purification fractions by SDS-polyacrylamide gel electrophoresis is shown in Fig. 4*A*. This gel was scanned with a densitometer to obtain the pattern shown in Fig. 4*B*. By integrating the peaks in Fig. 4*B* the purity of photolyase at each purification step was calculated. The results are summarized in Table I. From 17 g of cells we obtained 7.5 mg of photolyase of greater than 95% purity at 5.4% yield. In this particular preparation the final fraction contained some minor contaminants each at less than 1%. In other

TABLE I

*Purification of E. coli DNA photolyase*

| Fraction | Volume | Total protein | Photolyase[a] | | Purification factor | Yield |
|---|---|---|---|---|---|---|
| | *ml* | *mg* | % | *mg* | | % |
| 1. Cell-free extract | 100 | 860 | 15.3 | 132 | 1 | 100 |
| 2. Ammonium sulfate | 130 | 676 | 16.8 | 114 | 1.2 | 90 |
| 3. Phenyl Sepharose 1 | 40 | 200 | 36.4 | 73 | 2.5 | 52.7 |
| 4. Hydroxylapatite | 50 | 26 | 90 | 23 | 6 | 16.9 |
| 5. DEAE-cellulose | 50 | 25 | 93 | 23 | 6.4 | 16.6 |
| 6. DNA-cellulose | 6 | 9.8 | >95 | 9.8 | 6.8 | 7.1 |
| 7. Phenyl Sepharose 2 | 4 | 7.5 | >95 | 7.5 | 6.8 | 5.4 |

[a] Determined by densitometric scanning of Coomassie blue-stained SDS-polyacrylamide gels.

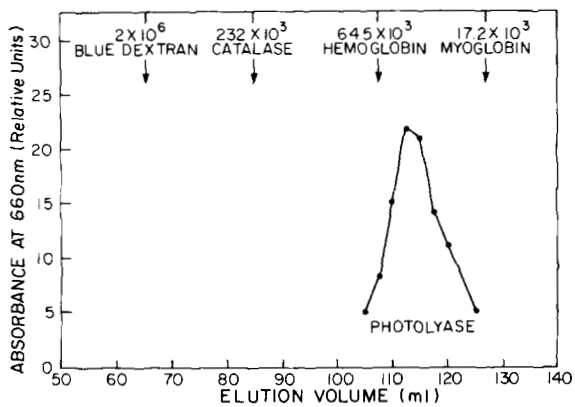


FIG. 5. **Gel filtration chromatography of photolyase on Ultrogel ACA44.** The positions of the markers (*arrows*) were determined spectrophotometrically; the molecular weight of each marker is indicated. To determine the position of photolyase the column fractions were analyzed an SDS-polyacrylamide gels and those fractions that contained photolyase were scanned with a densitometer using 660 mm of soft laser light.

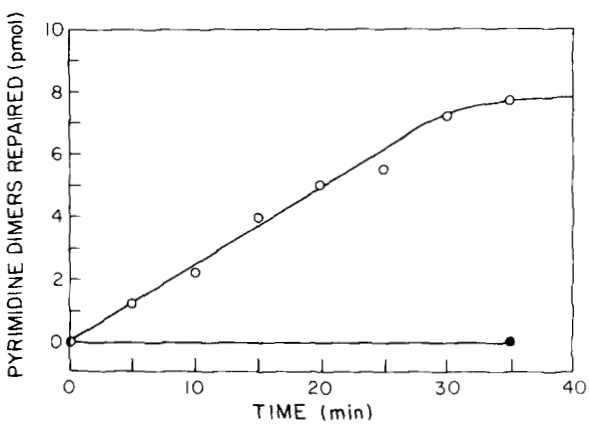


FIG. 6. **Repair of UV-irradiated pBR322 by photolyase.** The reaction mixture contained 9.8 pmol of lethal UV lesions, 8.8 pmol of which are assumed to be pyrimidine dimers and 0.1 pmol of photolyase. ●, not exposed to photoreactivating light; O, exposed to photoreactivating light.

preparations we have obtained photolyase free of any detectable (by Coomassie blue staining) contaminants (Fig. 4*A*, F7′).

### *Molecular Weight of the Native Protein*

The molecular weight of native photolyase was determined by gel filtration chromatography on an ACA44 column. The column (1.6 cm × 90 cm) was equilibrated with Buffer A containing 100 mM KCl; 125 μg of photolyase from Fraction VII were loaded onto the column with the appropriate $M_r$ markers, and the column was developed with the same buffer. The result is shown in Fig. 5. Photolyase elutes as a single peak of $M_r$ 49,000. Since this also is the $M_r$ obtained from SDS-polyacrylamide gels (3) (see also Fig. 4*A*), we conclude that the native enzyme is a monomer of a $M_r$ 49,000 polypeptide.

TABLE II

*The two photolyase activities in E. coli*

| Enzyme | Photolyase F | Photolyase R[a] |
|---|---|---|
| Gene | *phr* at 16.2 min | At *gal-attλ* interval |
| Phenotype of mutant | Photoreactivation-less | Photoreactivable |
| $M_r$ | 53,994[b] | 36,800 |
| Trp per molecule | 15[c] | None |
| Cofactor | FAD | RNA |
| Turnover No. (pyrimidine dimers/min) | 2.4 | $2.4 \times 10^{-3}$ |

[a] Based on data in Refs. 10 and 11.

[b] Based on the sequence of *phr* gene in the accompanying paper (8). The $M_r$ calculated from SDS-polyacrylamide gels is 49,000 (3).

[c] Based on sequence data in the accompanying paper (8).

### *Activity of the Purified Enzyme*

The activity of purified photolyase was tested by a transformation assay using UV-irradiated pBR322 as substrate and *E. coli* CSR603 as a host. The result is shown in Fig. 6. As expected the enzyme acts on UV-irradiated DNA only in the presence of photoreactivating light. The enzyme has no detectable endo- or exonuclease activity in the presence or absence of light (data not shown) and has no effect on transforming efficiency of nonirradiated DNA (2, 3). From the data in Fig. 6 we calculate a specific activity of $4 \times 10^6$ units/mg of enzyme and a turnover rate of 2.4 dimers/photolyase molecule/min.

## DISCUSSION

We have constructed a *tac-phr* plasmid that enabled us to amplify *E. coli* DNA photolyase approximately 15,000-fold (3). This in turn has made it possible, using the procedures described above, to obtain several milligrams of highly purified enzyme. Both the native and denatured forms of the enzyme exhibit an apparent $M_r$ of 49,000, in good agreement with the $M_r$ of 53,994 predicted from the gene sequence, as described in the accompanying paper (8); thus, active photolyase is a monomer. Using a transformation assay we have demonstrated that the purified enzyme exhibits properties expected for a photolyase, *i.e.* repair of UV-irradiated DNA only in the presence of photoreactivating light; furthermore the turnover rate of 2.4 dimers/photolyase molecule/min which we obtained with purified enzyme is in reasonable agreement with the turnover rate of 4.5 dimers/molecule/min obtained from *in vivo* experiments on *E. coli* $B_{s-1}$ by Harm (9).

It is worth mentioning that another protein having photoreactivating activity has been purified from *E. coli* to apparent homogeneity and has been found to carry an RNA co-factor (10, 11). For the sake of comparison we call these proteins photolyase F (for flavoprotein, the enzyme dealt with in this paper) and photolyase R (for RNA), respectively. A comparison of some of the properties of the two enzymes is given in Table II. It is apparent from this comparison that the two enzymes are different and that photolyase F is the major photolyase in *E. coli*. In fact, since this enzyme is 1,000-fold more active than photolyase R, it is conceivable that photo-

lyase R preparations consist of a $M_r$ = 35,000 protein of unknown function contaminated at a level of about 0.1% with photolyase F. However amplification of the $M_r$ = 35,000 protein by genetic means is reported to accompany a similar increase in photoreactivation activity. Clearly further work is needed to resolve this issue.

We have previously shown that purified photolyase is a flavoprotein having a noncovalent FAD moiety (2). The availability of large quantities of pure protein will help in elucidating the function of FAD in photoreactivation as well as the sites of interaction of dimer-containing DNA and photolyase. These studies are in progress.

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