Stimulation of *Escherichia coli* DNA Photoreactivating Enzyme Activity by Adenosine 5′-Triphosphate†

Prasad Koka*

ABSTRACT: A purification procedure consisting of Biorex-70, single-stranded DNA–agarose, and ultraviolet (UV) light irradiated DNA–cellulose chromatography has been adopted for the *Escherichia coli* photoreactivating enzyme. The purpose of this purification was to obtain enzyme preparations that are free of extraneous nucleic acid or nucleotides. The purification yields high specific activities (75 000 pmol h^{-1} mg^{-1}) with a 50% recovery. Enzyme preparations have also been obtained from UV-irradiated DNA–cellulose by exposure to visible light. These enzyme preparations contain oligoribonucleotides, the largest found to be 26 nucleotides in length in relation to DNA size markers. However, the oligoribonucleotides associated with the enzyme are not essential for enzymatic activity. When the enzyme is preincubated with exogenous ATP for 4–10 h at 3 °C, a 10-fold stimulation in the enzyme activity has been observed. It has been determined by polyacrylamide gel electrophoresis and high-voltage diethylaminoethyl paper electrophoresis that the light-released enzyme samples from a preincubated and washed mixture of the enzyme, [γ-^{32}P]ATP, and UV-irradiated DNA–cellulose contained exogenous [γ-^{32}P]ATP. [γ-^{32}P]ATP eluted with the enzyme-containing fractions when subjected to Bio-Gel P-30 chromatography. GTP caused a slight enhancement of the enzyme activity while ADP strongly inhibited photoreactivation, at the same concentration and conditions as those for ATP. Higher (×5) concentrations of ADP and adenosine 5′-(β,γ-methylenetriphosphate) totally inhibited the enzyme activity. Dialysis of a photoreactivating enzyme preparation against a buffer solution containing 1 mM ATP caused a 9-fold stimulation of the enzyme activity. In addition, there is an apparent hydrolysis of ATP during photoreactivation as measured by the release of ^{32}P from [γ-^{32}P]ATP.

Many types of cells that are irradiated with ultraviolet (UV)[1] light (220–320 nm) and subsequently exposed to visible light (300–600 nm) enhance their survival in comparison to those which are not illuminated with visible light. This phenomenon termed photoreactivation has been shown to be enzymatically mediated (Goodgal et al., 1957). The irradiation of DNA by UV light results in the formation of dimers between adjacent pyrimidines on the same DNA strand (Setlow, 1963). These dimers are the only substrate for the photoreactivating enzyme (Setlow & Setlow, 1963). The enzyme binds to the DNA (Rupert, 1962) and repairs the dimers by the action of visible light (Setlow et al., 1965; Cook, 1967). Photoreactivating enzymes have been isolated from many sources [for a review, see Sutherland (1981)]: *Escherichia coli* (Rupert, 1960; Sutherland et al., 1973; Snapka & Sutherland, 1980; Sancar et al., 1983; Yamamoto et al., 1983), yeast cells (Rupert, 1960), bakers' yeast (Minato & Werbin, 1971; Boatwright et al., 1975), *Streptomyces griseus* (Eker & Fichtinger-Schepman, 1975), *Anacystis nidulans* (Saito & Werbin, 1970), marsupials (Cook & Regan, 1969), human leukocytes (Sutherland, 1974), and cultured cells of *Drosophila melanogaster* (Beck, 1982).

Two *phr* genes have been mapped on the *E. coli* chromosome, one at 15.9 min (Sancar & Rupert, 1978a) and the other at 17 min (Sutherland et al., 1971). These genes have been designated *phr*A (17 min) and *phr*B (15.9 min) by Sutherland & Hausrath (1979), and the gene products have been amplified (Sutherland et al., 1972; Sancar et al., 1983). The *phr*B gene (15.9 min) has been recently cloned into plasmid, pMB9 (Sancar & Rupert, 1978b), and a *tac* vector (Sancar et al., 1983) which amplified the enzyme levels. A *phr* gene has also been cloned into pBR322 and the gene product amplified (Yamamoto et al., 1983). Snapka & Sutherland (1980) obtained a molecular weight of 35 000 for the amplified *E. coli* *phr*A enzyme. Sancar et al. (1983) identified and isolated an amplified enzyme with a molecular weight of 49 000. Sutherland et al. (1972) mapped the enzyme gene between *gal* and *att*λ on the *E. coli* chromosome. Cells with Δ(*gal-att*λ) contained 20% of normal enzyme level and activity (Sutherland & Hausrath, 1979). Δ(*gal-att*λ) mutants were found to be photoreactivable also by Sancar & Rupert (1978a) and by Youngs & Smith (1978). But a mutation in the *phr* gene locus at 15.9 min caused the absence of the 49 000-dalton protein and lacked photoreactivability (Sancar et al., 1983). Sancar et al. (1983) proposed that *E. coli* produces two photoreactivating enzymes, a major one encoded by the gene at 15.9 min and a minor one encoded by the gene between *gal–att*λ. They also proposed that the activity of the latter enzyme is detectable only when genetically amplified.

An *E. coli* enzyme was isolated and purified (Sutherland et al., 1973; Snapka & Sutherland, 1980) by the induction of a strain lysogenic for a transducing phage carrying the *phr*A gene (Sutherland et al., 1972). Snapka & Fuselier (1977) proposed an adenine-containing cofactor for this enzyme. Preparations of the enzyme were shown to be associated with oligoribonucleotides (Snapka & Sutherland, 1980; Koka & Sutherland, 1980, 1982; Cimino & Sutherland, 1982). [Enzyme preparation from the cultured cells of *D. melanogaster* contained small RNA (Beck, 1982)]. Sutherland (1981) proposed that each enzyme molecule is associated with one

† From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973. *Received November 15, 1983.* This work was supported by a grant from the National Cancer Institute (CA23096) and by a grant from the U.S. Department of Energy to Dr. Betsy M. Sutherland.

* Address correspondence to this author at E17-340, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.

[1] Abbreviations: UV, ultraviolet; ssDNA, single-stranded deoxyribonucleic acid; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GMP, guanosine 5′-monophosphate; PSEG buffer, potassium phosphate + sodium chloride + EDTA + glycerol; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-2914$01.50/0

Table I: Purification of the Photoreactivating Enzyme from *Escherichia coli*

photoreactivating enzyme fraction	total protein (mg)	total absorbance at 260 nm	sp act. ($\times 10^3$ pmol h^{-1} mg^{-1})	total activity ($\times 10^4$ pmol h^{-1} mL^{-1})	% recovery
dialyzed fraction III	10	225	3.8	3.8	100
Biorex-70 chromatography	1.9	70	10.6	2.0	52
ssDNA–agarose chromatography	1.3	57	17.3	2.2	58
UV-irradiated DNA–cellulose chromatography, elution with 0.6 M potassium phosphate + 2.0 M NaCl + 1 mM EDTA	0.26	<1	74.8	2.0	52

oligoribonucleotide serving as a cofactor. Cimino & Sutherland (1982) found spectroscopic evidence for the secondary structure of the oligoribonucleotides associated with an enzyme preparation.

In order to investigate the possible role of RNA in the functioning of the *E. coli* enzyme, it was necessary to develop purification procedures which would yield enzyme preparations free of extraneous nucleic acid and nucleotides. Using the enzyme preparations obtained by these purification methods, the possible involvement of RNA in the catalytic activity of the enzyme was investigated. During this investigation, it was found that the *E. coli* enzyme showed enhanced photoreactivating activity when preincubated with ATP. Evidence was found for the utilization of ATP for the photorepair process of the pyrimidine dimer containing DNA by the enzyme and that ATP is not merely an allosteric effector of the enzyme. This finding delineates a role for ATP in the molecular mechanism of photoreactivation.

Experimental Procedures

Purification of Photoreactivating Enzyme from E. coli. The initial purification steps to obtain the 60% ammonium sulfate precipitate of the enzyme and its resuspension in buffer E containing 40% glycerol (fraction III) were as described by Snapka & Sutherland (1980).

Of the above enzyme fraction, 0.75 mL was dialyzed at 4 °C for 7 h against 5 L of PSEG buffer (25 mM potassium phosphate, pH 6.7, 70 mM NaCl, 1 mM EDTA, and 20% glycerol) with changes of 700 mL of buffer every hour. There was no loss in enzyme activity after the dialysis.

Of the dialyzed enzyme preparation, 1.5 mL was applied to and eluted from a 3.0-mL Biorex-70 (Bio-Rad) column (5-mL syringe with an 18-gauge 1.5-in. needle) that was equilibrated with PSEG buffer. The eluting material was monitored at 254 nm by using an ISCO Model UA-5 UV monitor with a type 6 dual-beam optical unit. Fractions of 1.2 mL were collected and assayed for enzyme activity. Biorex-70 chromatography (Figure 1) resulted in the removal of almost 80% of the extraneous protein and 70% of the nucleic acid (Table I). Chromatographic conditions were adjusted such that the enzyme would not bind to the Biorex resin but other proteins did bind to the resin. Nucleic acids did not bind to the column, but there was a separation of nucleic acids on the column with the larger nucleic acids following the smaller nucleic acids in the enzyme fractions. This was observed by the 260-nm absorbance (Figure 1) and by 5′-end labeling of the nucleic acids from different fractions of Biorex-70 elution followed by 20% polyacrylamide sequencing gel electrophoresis (data not included).

It was found that most of the DNA binding proteins that were present in the enzyme fractions from the Biorex chromatography step bound preferentially to single-stranded DNA. The ssDNA–agarose chromatography was therefore used to remove the DNA binding proteins present in the enzyme

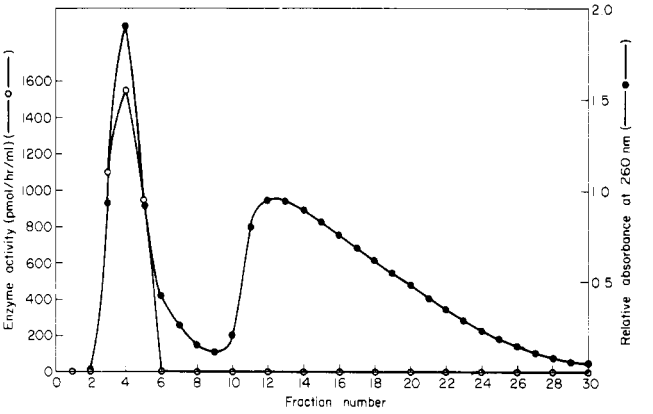

FIGURE 1: Biorex-70 chromatography of the ammonium sulfate fraction of the photoreactivating enzyme dialyzed against the PSEG buffer.

preparations from the Biorex column. Two milliliters of the enzyme from Biorex-70 chromatography was passed through a 2.0-mL single-stranded DNA–agarose (Bethesda Research Labs) column (3-mL syringe) that was equilibrated with PSEG buffer. Conditions for running the column were such that only the DNA binding proteins would bind to the ssDNA–agarose and minimal binding, if any, of the enzyme to the resin occurred.

The fractions from the ssDNA–agarose chromatography which showed enzyme activity were pooled and loaded onto a 4.0-mL UV-irradiated DNA–cellulose column (5-mL syringe) that was equilibrated with PSEG buffer. Loading was at a flow rate of 5 mL/h under the conditions of yellow light illumination. The material eluting from this column was passed through the sample cell of the optical unit of the UV monitor. A reference 4.0-mL column of cellulose was also equilibrated with the same buffer, and the eluting buffer was passed through the reference cell of the optical unit. The length of the tubings and the hydrostatic pressure were the same for both of the columns with a common buffer reservoir. Fractions of 1.2 mL were collected from the sample column. The running buffer was changed to one of a higher ionic strength (0.3M potassium phosphate, pH 7.2, 1.0 M NaCl, 1 mM EDTA, and 20% glycerol) after the enzyme was applied and the columns were thoroughly washed with PSEG buffer. Fractions were collected continuously during the washings with PSEG buffer after the enzyme was loaded onto the column and during the elutions and washings from the columns with the higher ionic strength buffer. The reservoir buffer was then replaced with the elution buffer (0.6 M potassium phosphate, pH 7.2, 2.0 M NaCl, and 1 mM EDTA), and fractions were collected as before. The collected fractions were assayed for enzyme activity. The use of two columns, a sample column and a reference column, for this chromatography step was to facilitate the detection of very small sample optical densities at 254 nm. It is recommended that these high-salt buffers be stored at about 10–15 °C instead of 3 °C to prevent salt

precipitation and that the column chromatography using these buffer solutions be done at 15 °C to avoid salt precipitation and freezing of the material passing through the columns.

The UV-irradiated DNA–cellulose was prepared from acid-washed cellulose and calf thymus DNA by the method of Alberts et al. (1973). The dried UV-irradiated DNA–cellulose resin was thoroughly washed with the elution buffer prior to equilibration with PSEG buffer. The binding capacity of the UV-irradiated DNA to the cellulose was about 1.5 mg of DNA/g of cellulose.

Enzyme from UV-Irradiated DNA–Cellulose by Visible Light Illumination. One-milliliter portions of the washed resin were added to 14 × 60 mm centrifuge tubes separately and equilibrated with PSEG buffer by repeated additions of the buffer and centrifugations at 10000 rpm for 5–10 min in a Du Pont SE-12 rotor. The photoreactivating enzyme from the Biorex-70 and/or ssDNA–agarose chromatography was added (about 0.2 mg of protein/mL of resin in the presence of 20 pmol of ATP/10 μL or 5–6 μg of Biorex enzyme) and allowed to stand overnight on ice. The resins in the tubes were then thoroughly washed with the PSEG buffer by using the above procedure. One tube was exposed to visible light illumination (from a Sylvania white light bulb) at 3 °C for 4 h, with frequent shaking. A control tube identical with the one under visible light was kept in the dark at 3 °C for 4 h, with frequent shaking. The tubes were then centrifuged, and the supernatant was assayed for enzyme activity.

ATP-Containing Enzyme Fractions. The enzyme was incubated with ATP or [γ-^{32}P]ATP (see Results for conditions) after elution from Biorex-70 or before binding to the affinity resin, UV-irradiated DNA–cellulose. In the case of the Biorex-70-eluted enzyme after its incubation with [γ-^{32}P]ATP, the [γ-^{32}P]ATP-bound enzyme was passed through a Bio-Gel P-30 column to separate the free [γ-^{32}P]ATP. The ATP-containing enzyme that eluted in the void volume of Bio-Gel P-30 was assayed for activity. If nonradioactive ATP was used, then the ATP–enzyme mixture was assayed for photoreactivation without gel filtration on Bio-Gel P-30. Whenever necessary, the [γ-^{32}P]ATP-containing enzyme (after Biorex-70 chromatography) was subjected to Bio-Gel P-30 chromatography, allowed to bind to UV-irradiated DNA–cellulose, and then thoroughly washed with PSEG buffer. The enzyme was then eluted from the UV-irradiated DNA–cellulose by visible light illumination and centrifugation. The released ATP-containing enzyme was then assayed for enzyme activity. These enzyme samples contained a certain amount of [γ-^{32}P]ATP that dissociated from the enzyme material bound to UV-irradiated DNA–cellulose (see Results).

Photoreactivating Enzyme Assay. The assay for photoreactivation was as described by Sutherland & Chamberlin (1973) and Snapka & Sutherland (1980) except that the substance dimer concentrations were increased to a final concentration of 2×10^{-7} M or higher. This modification was partly responsible for the enhanced specific activities. To ensure proper digestion of the increased amount of the UV-irradiated DNA substrate, a 2-fold increase in the concentration of the digest enzymes was made.

Protein Assays. The amount of protein in enzyme fractions was determined by the Lowry (Lowry et al., 1951) or Bio-Rad (Bradford, 1976) assay. Ovalbumin was used as the protein standard. The estimates of specific activities (Table I) were based on the protein content determined by the micro-Lowry method.

5′-End Labeling of the Nucleic Acids. The nucleic acids present in the enzyme samples were labeled at their 5′ ends with ^{32}P by [γ-^{32}P]ATP (2900 Ci/mmol) (New England Nuclear) and T4 polynucleotide kinase (P-L Biochemicals) (Richardson, 1972). The labeling reaction mixture in each tube consisted of 10 mM $MgCl_2$, 1 mM DTT, 2 pmol/μL (3 μCi/μL) [γ-^{32}P]ATP, and 0.02–0.03 unit of polynucleotide kinase. The 5′-end labeling was done at 4 °C for 5 h. It was found that no prior treatment of the RNA with alkaline phosphatase was necessary.

The 5′-end-labeled nucleic acids were also subjected to alkaline hydrolysis (0.4 N NaOH, 18 h, 37 °C) and digestion with ribonuclease T_1 (1 mg/mL) and pancreatic ribonuclease (1 mg/mL) [Sephadex G-75 superfine fraction (gift from J. J. Dunn)] at 37 °C for 2–5 h.

DNA size markers obtained from J. J. Dunn were used to estimate the size of the nucleic acids associated with the enzyme. The markers were generated from the adenovirus 2 genome by the Maxam & Gilbert (1980) technique after the DNA was cut with the appropriate restriction endonucleases and labeled with ^{32}P.

Sequencing Gels. Polyacrylamide–urea sequencing gels (8% or 20%) were run in TBE buffer (0.045 M Tris–0.04 M borate–1 mM EDTA) at 1 kV for 2.5 h by using the method of Maxam & Gilbert (1980). The gels were then covered with a plastic wrap and exposed to Dupont Cronex 4 X-ray film at −70 °C. The developed films were used to determine the size of the RNA species.

Some of the 5′-end-labeled nucleic acids were purified by gel electrophoresis by excising the bands containing nucleic acid and eluting the material in 0.2–0.3 M NaCl by diffusion overnight at 37 °C. The material was then diluted, loaded onto a small DEAE-cellulose column, washed with 10 mM Tris (pH 8.0) buffer containing 1 mM EDTA and then with water, and eluted with 10% triethylammonium bicarbonate, pH 8.5. The material was then lyophilized by repeated additions of water.

Results

Enzyme Purification. The enzyme was subjected to the described purification procedure to obtain preparations that were better separated from extraneous protein and nucleotides and nucleic acid. Table I gives the enzyme purification steps and the specific activities obtained by the procedures adopted. The specific activities noted are the highest values obtained for the *E. coli* photoreactivating enzyme. The specific activity of the enzyme that was released by light from the UV-irradiated DNA–cellulose was found to be 96800 pmol h^{-1} mg^{-1}.

Table I also indicates that this purification procedure results in the removal of greater than 90% of the protein and 99% of the nucleic acid (or 260-nm-absorbing material). The results strongly suggest that separation of most of the nucleic acid associated with the protein present in enzyme preparations, fraction III (Snapka & Sutherland, 1980), does not result in loss of enzyme activity. It was found by 5′-end ^{32}P labeling of the nucleic acids of the enzyme that were run on a 20% polyacrylamide sequencing gel that most of the large nucleic acids present in fraction III were separated by Biorex-70 chromatography (data not included).

Effect of Pancreatic Ribonuclease on Enzyme Activity. In order to determine whether the RNA associated with the enzyme is essential for its activity, the effect of ribonuclease on enzyme activity was tested. It was previously reported that the activity of a partially purified photoreactivating enzyme is sensitive to digestion by pancreatic ribonuclease (Koka & Sutherland, 1980). Since it has been observed in this investigation (see below for details) that exogenous ATP stimulated the enzyme activity, these experiments were repeated (Table

Table II: Effect of Pancreatic Ribonuclease on the Activity of ATP-Containing *E. coli* Photoreactivating Enzyme Obtained from UV-Irradiated DNA–Cellulose by Photoreactivation

pancreatic ribonuclease (ng)	UV-irradiated DNA substrate (μg)	sp act. (nmol h^{-1} mg^{-1})
0	5	240
1.0	5	24
2.5	12.5	72
5.0	25	133
5.0	5	*a*

[a] Interference with the photoreactivating enzyme assay at this ratio of ribonuclease to UV-irradiated DNA substrate.

II) with the ATP-containing photoreactivating enzyme that was obtained from UV-irradiated DNA–cellulose by visible light illumination. Not only the specific activity of this enzyme preparation was much higher but also the amount of nucleic acid [as measured by the absorbance at 260 nm (Table I)] in relation to the protein was much lower than that of other enzyme preparations. The presence of excess ATP should also minimize the binding of other nucleotides to the enzyme.

Table II shows the effect of pancreatic ribonuclease on photoreactivation. The ATP-containing enzyme obtained from UV-irradiated DNA–cellulose by visible light was first preincubated with pancreatic ribonuclease for 15 min at 37 °C in the presence of the pyrimidine dimer containing DNA substrate and then exposed to photoreactivating light. The amount of photoreactivating enzyme present in these experiments was 120 ng and the DNA substrate, 5 μg or higher. The effect of the addition of 1 ng of RNase without increasing the amount of DNA substrate suggests an apparent inhibition of photoreactivating enzyme activity by RNase treatment. The effect of increasing the amount of RNase without increasing the amount of DNA substrate suggested that RNase is binding to the DNA substrate of the photoreactivating enzyme and is therefore causing interference with the enzyme assay at the DNA digestion stage. When the amounts of both the RNase and the DNA substrate were proportionately increased in the presence of the same amount of photoreactivating enzyme, there was a proportionate trend toward the restoration of the photoreactivating enzyme activity. Greater amounts of DNA present an opportunity for the RNase to bind to additional DNA regions, causing interference with the photoreactivating enzyme assay, and also, as a result of binding to DNA, less RNase may be available to digest the RNA associated with the enzyme. However, greater amounts of DNA would also mean additional dimer regions being available for photoreactivation despite the binding of RNase to additional DNA. Thus, the ratio of number of dimer regions to number of enzyme molecules increases when the amounts of RNase and DNA substrate are proportionally increased.

RNA Associated with the Enzyme. An attempt was made to determine whether any unique RNA species or several RNAs of a similar size class were found to be associated with the enzyme. The electrophoresis of the 5′-end ^{32}P-labeled nucleic acids associated with the photoreactivating enzyme obtained from the UV-irradiated DNA–cellulose by visible light illumination was carried out on 20% and 8% polyacrylamide gels. These nucleic acids were electrophoresed with DNA size markers to estimate their size. The largest observable nucleic acids were of the size 24–26 nucleotides (data not shown). However, the sizes of the nucleic acids seem to vary with different preparations of the enzyme, and the RNA present is not homogeneous in nature. The nucleic acids were susceptible to digestion by ribonuclease T_1 and pancreatic

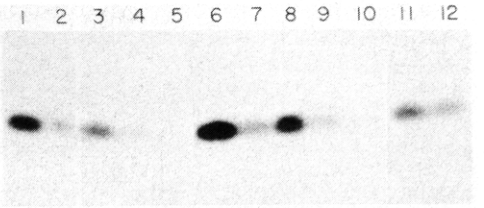

FIGURE 2: Presence of [γ-^{32}P]ATP in photoreactivating enzyme fractions as determined by polyacrylamide gel electrophoresis. The enzyme samples were incubated with [γ-^{32}P]ATP and the resin followed by thorough washings with the buffer and subsequent electrophoresis. Band 1, photoreactivated (2 h) sample consisting of [γ-^{32}P]ATP (25 μCi, 8 pmol), enzyme (50 μg from Biorex-70), and the resin (UV-irradiated DNA–cellulose) (50 μL); band 2, same as band 1 sample but no photoreactivation; band 3, same as for band 1 but with 100 μL of resin; band 4, same as for band 2 but with 100 μL of resin; band 5, light-exposed (2 h) sample consisting of [γ-^{32}P]ATP and the resin (50 μL) but no enzyme; band 6, photoreactivated (4 h) sample consisting of [γ-^{32}P]ATP, enzyme, and the resin (50 μL); band 7, same as band 6 sample but no photoreactivation; band 8, same as for band 6 but with 100 μL of resin; band 9, same as for band 7 but with 100 μL of resin; band 10, same as for band 5 but exposed to light for 4 h; band 11, same as for band 8 except the resin DNA–cellulose was not UV irradiated; band 12, same as for band 11 except the sample was not photoreactivated. Bands 11 and 12 are taken from a different autoradiogram.

ribonuclease. It is possible that these ribonucleotides are a contamination from the calf thymus DNA used to prepare the UV-irradiated DNA–cellulose, but these enzyme preparations seem to be lacking oligodeoxyribonucleotides.

Presence of Exogenous ATP in Photoreactivating Enzyme Fractions As Determined by Gel Electrophoresis. The results depicted in Figure 2 show that [γ-^{32}P]ATP is present in the fractions that contain the photoreactivating enzyme activity. As described under Experimental Procedures, the [γ-^{32}P]-ATP-containing enzyme samples that were bound to UV-irradiated DNA–cellulose and later subjected to visible light illumination were subjected to electrophoresis on a 20% polyacrylamide–urea gel. Control samples that were eluted by centrifugation from (1) the [γ-^{32}P]ATP-containing enzyme that was incubated with UV-irradiated DNA–cellulose but not exposed to visible light, (2) the [γ-^{32}P]ATP-containing enzyme incubated with cellulose to which no UV-irradiated DNA was bound, (3) [γ-^{32}P]ATP incubated with UV-irradiated DNA–cellulose but without enzyme, and (4) [γ-^{32}P]-ATP-containing enzyme incubated with DNA–cellulose the DNA of which was *not* UV irradiated were also subjected to gel electrophoresis. The single radioactive band from the gel seen on the film was identified as ATP by high-voltage (4.5 kV) DEAE and Whatman 3MM paper and gel electrophoresis of the light-released [γ-^{32}P]ATP-containing enzyme sample and free [γ-^{32}P]ATP with and without alkaline phosphatase treatment (37 °C, 1 h).

The enzyme was first incubated with [γ-^{32}P]ATP under optimum conditions (see later details and figures) at 3 °C and then allowed to bind to UV-irradiated DNA–cellulose by incubation overnight at 3 °C. The control samples described above were also incubated separately. The resins were then washed thoroughly with PSEG buffer by repeated centrifugations. After visible light illumination at 3 °C for 1–4 h, the samples were subjected to 20% polyacrylamide gel electrophoresis along with samples that were kept in the dark. The [γ-^{32}P]ATP band seen on the gel was due to a certain amount of dissociation of [γ-^{32}P]ATP from the enzyme material that was bound to UV-irradiated DNA–cellulose in the samples that were being illuminated or kept in the dark. In the samples wherein the incubation mixture consisted of [γ-^{32}P]ATP and

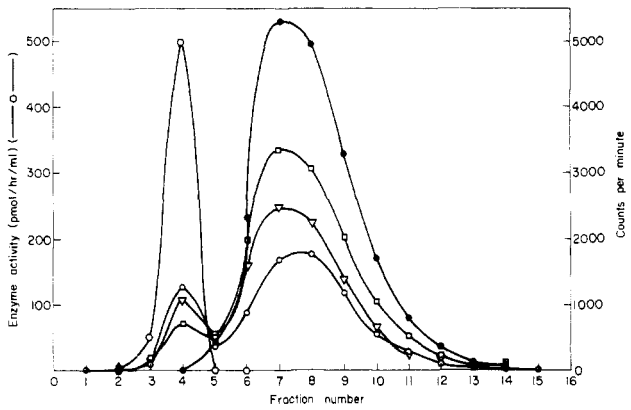

FIGURE 3: Binding of [γ-^{32}P]ATP to the photoreactivating enzyme-containing material as shown by gel filtration on Bio-Gel P-30. (○) elution of Biorex-70 enzyme (75 μg/150 μL) activity; (●) elution of [γ-^{32}P]ATP (8 pmol/5 μL) + PSEG buffer (150 μL); (○) elution of [γ-^{32}P]ATP (8 pmol/5 μL) + enzyme (75 μg/150 μL); (▽) elution of [γ-^{32}P]ATP (8 pmol/5 μL) + enzyme (50 μg/100 μL) + buffer (50 μL); (□) elution of [γ-^{32}P]ATP (8 pmol/5 μL) + enzyme (25 μg/50 μL) + buffer (100 μL). The [γ-^{32}P]ATP and enzyme mixtures were incubated for 4.5 h at 3 °C prior to Bio-Gel P-30 chromatography.

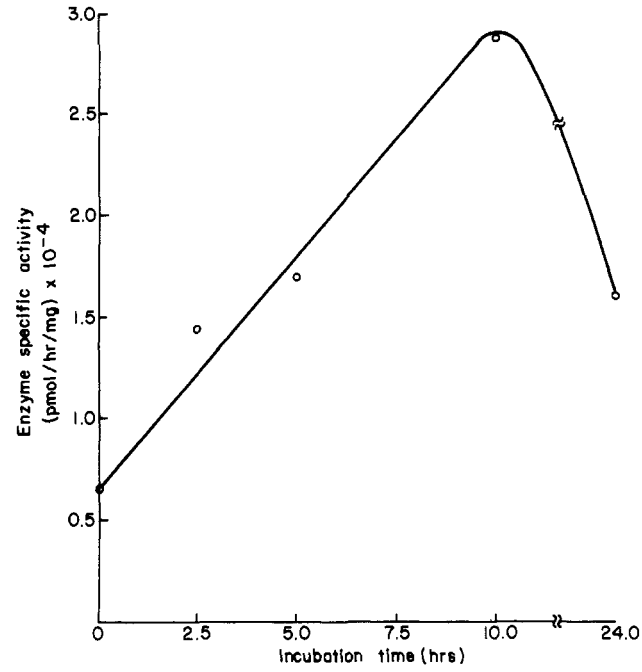

FIGURE 4: Photoreactivating enzyme activity as a function of the incubation time of the Biorex-70 enzyme (5 μg) with ATP (20 pmol).

the UV-irradiated DNA–cellulose (no enzyme), a radioactive band in the position corresponding to that of [γ-^{32}P]ATP was barely visible on the gel. In the samples wherein the incubation mixture consisted of [γ-^{32}P]ATP, enzyme, and DNA–cellulose that was not UV irradiated, a radioactive band was visible but with a lower intensity in comparison to the samples eluted from UV-irradiated DNA–cellulose. These results indicate that the binding of ATP to the UV-irradiated DNA–cellulose is contingent upon the presence of the photoreactivating enzyme preparations, since there was no direct binding of the ATP to the UV-irradiated DNA–cellulose as indicated by the absence of [γ-^{32}P]ATP in the samples that did not contain the enzyme. They also suggest a greater amount of [γ-^{32}P]ATP dissociation from the enzyme material bound to UV-irradiated DNA compared to DNA without pyrimidine dimers, which is consistent with a greater amount of enzyme binding to DNA in the presence of pyrimidine dimers. There was no detectable enzyme activity in control samples in which the enzyme was present in the incubation mixture.

It was also determined by the same technique by the presence of [γ-^{32}P]ATP in the enzyme samples eluted after washing the resin, illumination, and centrifugation that [γ-^{32}P]ATP could bind to the enzyme even after the enzyme was first incubated with UV-irradiated DNA–cellulose at 3 °C for about 4 h to form the enzyme–substrate complex followed by incubation overnight with [γ-^{32}P]ATP added (data not shown). However, it is possible that without exogenous ATP, the enzyme–substrate complexes that were formed were due to the presence of endogenous ATP and additional enzyme–substrate complexes were formed as a result of the added [γ-^{32}P]ATP. The filter binding experiment (A. Shih, personal communication) in which the binding of the enzyme to ^{32}P-labeled UV-irradiated DNA was determined in the presence and absence of ATP also suggests that the binding is independent of the presence of ATP.

Binding of ATP to Photoreactivating Enzyme Containing Material As Determined by Gel Filtration. Figure 3 shows the binding of [γ-^{32}P]ATP to the photoreactivating enzyme samples from Biorex-70 by the technique of gel filtration on Bio-Gel P-30. The enzyme elutes in the void volume of the Bio-Gel P-30 column. This was verified by assaying the enzyme activity. Increasing amounts of the Biorex-70 enzyme fractions were incubated separately for 5 h at 3 °C with the same amount of [γ-^{32}P]ATP, both contained in the same total volume. Each was passed through a Bio-Gel P-30 column that was washed and equilibrated with PSEG buffer, and the radioactivity of eluting fractions was counted. The results indicated that increased amounts of [γ-^{32}P]ATP were associating with increasing amounts of enzyme in the samples that were loaded and eluted from the column. There was also a corresponding decrease in the free [γ-^{32}P]ATP. The sum of the [γ-^{32}P]ATP eluted free and with the enzyme is on the average about 70% of the [γ-^{32}P]ATP added to the enzyme. The 30% loss is possibly due to nonspecific binding of the [γ-^{32}P]ATP to the column and the resin. These results suggest a physical association between the enzyme and ATP.

Photoreactivating Enzyme Activity as a Function of the Time of Incubation with ATP. The enzyme activity (from Biorex-70) increased with increasing time of preincubation of the enzyme with ATP at 3 °C, attaining maximum stimulation of enzyme activity in about 10 h (Figure 4). At times, the maximum stimulation was achieved after an incubation time of 4–5 h. The concentration of the unlabeled ATP used (20 pmol/5–6 μg of the enzyme obtained by Biorex-70 chromatography) was such as to provide maximum stimulation of enzyme activity (see Figure 6). Preincubation with ATP seems to be necessary to achieve stimulation, and incubation for times longer than 12 h inhibits enzyme activity possibly due to the accumulation of ADP formed by hydrolysis of ATP in solution during preincubation. It is possible that different preparations of the Biorex enzyme require different lengths of time of preincubation with ATP.

Photoreactivating Enzyme Activity Is Increased in the Presence of Exogenous ATP. Figure 5 shows the enzyme (from Biorex-70) activity with or without incubation with ATP for 10 h at 3 °C. The ATP concentration, 20 pmol/5–6 μg of enzyme, was such as to yield maximum stimulation of enzyme activity (see later details). Though most often a 10-fold enhancement of enzyme activity by ATP (20 pmol/5 μg of protein) was achieved, lower levels of stimulation were also observed which was possibly due to the use of different enzyme preparations. The drop in activity when larger amounts of enzyme with ATP were examined is possibly be-

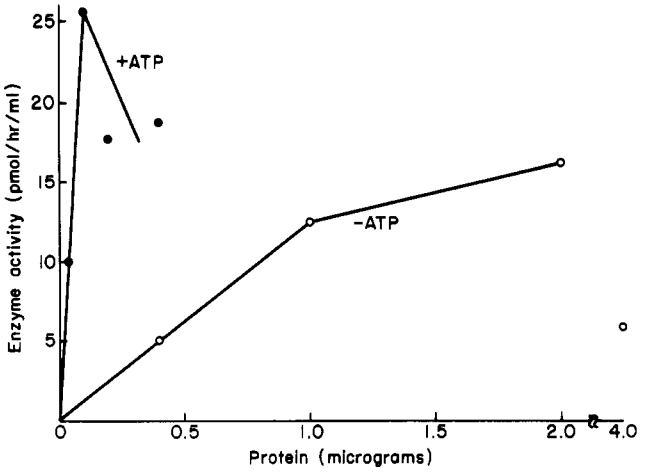

FIGURE 5: Photoreactivating enzyme (from Biorex-70) activity in the presence or absence of ATP. Samples of enzyme–ATP in the ratio of 5 μg to 20 pmol were incubated at 3 °C for 10 h. Enzyme samples without ATP were also incubated under the same conditions.

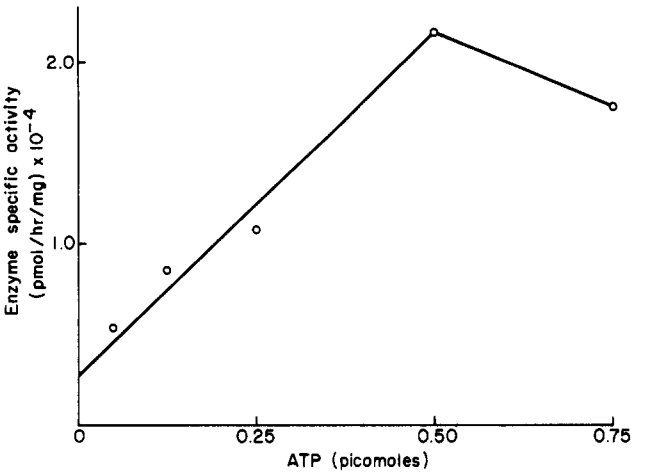

FIGURE 6: Enzyme specific activity as a function of ATP concentration. The enzyme–ATP samples were incubated for 10 h at 3 °C. Protein was measured by the Lowry method.

Table III: Amount of Photoreactivating Enzyme Protein Released from UV-Irradiated DNA–Cellulose by Visible Light Illumination in the Presence or Absence of Binding of [γ-^{32}P]ATP[a]

enzyme with or without ATP	enzyme protein released (μg)	activity ($\times 10^3$ pmol h^{-1} mL^{-1})	sp act. ($\times 10^4$ pmol h^{-1} mg^{-1})
with ATP	0.20	40	9.97
without ATP	0.05	9.7	9.68

[a] Fifty micrograms of enzyme and 1.8 pmol of [γ-^{32}P]ATP were preincubated with UV-irradiated DNA–cellulose at 3 °C overnight followed by further incubation with 200 pmol of ATP for 2–4 h and then thoroughly washed with the buffer prior to photoreactivation.

Table IV: Photoreactivating Enzyme (from Biorex-70 Chromatography) Activity in the Presence of ATP or Its Analogues[a]

analogue added	amount of analogue (nmol)	enzyme sp act. ($\times 10^3$ pmol h^{-1} mg^{-1})
none		10
ATP	0.02	51
GTP	0.02	13
GTP	1.00	8
β,γ-methylene-ATP	0.02	11
β,γ-methylene-ATP	1.00	0
ADP	0.02	2
ADP	1.00	0.7

[a] Protein was measured by the Lowry method.

cause of an insufficient amount of substrate and binding of the ADP produced (see later details) to the enzyme.

The enzyme obtained by ammonium sulfate precipitation was dialyzed against PSEG buffer with and without 1 mM ATP and then assayed for activity. The enzyme dialyzed against buffer containing ATP showed a 10-fold higher activity, with a specific activity of 5806 pmol h^{-1} mg^{-1} without ATP and 54 769 pmol h^{-1} mg^{-1} with ATP.

Influence of ATP on the Release of Photoreactivating Enzyme from UV-Irradiated DNA–Cellulose. The enzyme (50 μg) from the Biorex-70 chromatography step was incubated with UV-irradiated DNA–cellulose at 3 °C in the presence and absence of 200 pmol of ATP (including 1.8 pmol of [γ-^{32}P]ATP). The enzyme was then released by visible light from the UV-irradiated DNA–cellulose, and the fractions were assayed for the amount of protein (Table III). The exogenous ATP-containing enzyme that was released from the UV-irradiated DNA–cellulose contained 4 times more protein (40 μg/mL) than the enzyme sample in which no ATP was added or only endogenous ATP could have been present. Therefore, the activity (picomoles per hour per milliliter of protein) of the ATP-containing enzyme that was released by light was also 4 times greater than that of the enzyme without added ATP. However, the specific activity (picomoles per hour per milligram of protein) of the enzyme that was released by light from the substrate UV-irradiated DNA–cellulose with or without added ATP was the same. This indicates that the protein that was released by light from the UV-irradiated DNA was almost entirely photoreactivating enzyme and that in the presence of exogenous ATP more enzyme was released.

Photoreactivating Enzyme Activity as a Function of the Concentration of ATP or Its Analogues. The enzyme from the Biorex-70 chromatography step was incubated with increasing amounts of ATP and then assayed for activity. Figure 6 shows the effect of increasing concentrations of ATP on the enzyme activity; 5–6 μg (125 ng final amount) of this enzyme preparation yielded a 7-fold stimulation of activity in the presence of 20 pmol (0.5 pmol final amount) of ATP (or 2.5 nM ATP). The Biorex-70 enzyme concentration was about 18 nM on the basis of a molecular weight of 35 200 (Snapka & Sutherland, 1980).

Table IV shows the effect of ATP analogues on enzyme activity. ADP seems to be a strong inhibitor of enzyme activity. GTP caused a slight stimulation of enzyme activity. The enzyme activity in the presence of β-γ-methylene-ATP at a concentration of 20 pmol/5 μg of protein was unchanged, and higher concentrations of this analogue totally inhibited the enzyme activity.

ATP Hydrolysis. The above results showing the inhibition of the enzyme activity by ADP and β,γ-methylene-ATP suggest that the hydrolysis of ATP occurs during photoreactivation. Photoreactivating enzyme with bound [γ-^{32}P]ATP obtained after Biorex-70 and Bio-Gel P-30 chromatography described as above was incubated with the substrate, UV-irradiated DNA that was not radioactive. One control tube was kept in the dark for each tube that was provided with visible light illumination. The conditions were the same as those for the enzyme assay procedure (Sutherland & Chamberlin, 1973) except that the substrate pyrimidine dimer concentration was increased to 2×10^{-5} M. Following incubation of the enzyme–substrate mixture with or without light, 0.04 N HCl (10 μL) and 4% (w/v) Norit (2 mL) were successively added,

Table V: Amount of ^{32}P Released from [γ-^{32}P]ATP by Photoreactivation of UV-Irradiated DNA Substrate with the Enzyme (0.07 μg/μL) from Biorex-70 Chromatography

photoreactivating enzyme (μL)	^{32}P released from [γ-^{32}P]ATP (Δcpm)
25	25
50	60
100	150

and then the mixture was filtered through Millipore (0.45-μm size) filters by using a syringe. Equal amounts of the filtrate from each tube were counted on a scintillation counter for Cherenkov radiation. If the radioactivity (counts per minute) of the light-treated sample filtrate was greater than that of the dark-kept sample filtrate, this was taken as a measure of the ^{32}P released from the [γ-^{32}P]ATP–enzyme complex as a result of the hydrolysis of ATP. The amount of ^{32}P released from the [γ-^{32}P]ATP–enzyme complex following photoreactivation of the UV-irradiated DNA substrate is shown in Table V. The amount of ^{32}P released was in proportion to the amount of enzyme that was added to the substrate for photoreactivation. The results shown in Table V confirm those in Table IV suggesting hydrolysis of ATP during photoreactivation of the pyrimidine dimer containing DNA by the photoreactivating enzyme.

Discussion

The photoreactivating enzyme from *Escherichia coli* was subjected to the described purification procedure in order to free the enzyme from extraneous nucleic acid or nucleotide-containing material. The mono-, di-, tri-, or oligonucleotides found present in enzyme preparations could be generated by contaminating nucleases, and thus it was necessary to free the enzyme from both the contaminating nucleases and nucleotides or nucleic acids during the enzyme purification. The previous isoelectrofocusing step (Snapka & Sutherland, 1980) lasted several days and did not give satisfactory results at least with respect to the separation of large nucleic acids. Biorex-70 binds basic proteins such as ribonuclease. Biorex-70 chromatography in addition to the removal of several extraneous proteins also resulted in separation of a large amount of nucleic acid material present in enzyme samples. The enzyme was released from the UV-irradiated DNA–cellulose by a very high ionic strength buffer, suggesting a very tight binding to the substrate (dimer containing DNA). Previous reports made by others suggested using a lower ionic strength buffer than the elution buffer used in the procedure described in this paper. This may be due to the binding of the enzyme to non-dimer regions of the DNA, the release from which can take place with a lower ionic strength buffer. On the other hand, this may be due to the presence of two different enzymes in *E. coli* (Sancar et al., 1983). The purification resulted in a 50% recovery of the enzyme activity with specific activities 10–15 times greater than those previously reported. The amount of protein available after purification from the UV-irradiated DNA–cellulose (either by elution with high ionic strength buffer or by release by light exposure) was so labile and small in quantity that it was not feasible to estimate the molecular weight by gel filtration or gel electrophoresis.

The discovery of a second photoreactivating enzyme by Sancar et al. (1983) with a molecular weight of 49 000 and which is different from that of Snapka & Sutherland (1980) with a molecular weight of 35 000 raises the possibility of the presence of two different enzymes in *E. coli* (Sancar et al., 1983). In this paper, the effect of ATP on a photoreactivating enzyme has been dealt with. The stimulation of the enzyme activity by ATP was observed by the method of Sutherland & Chamberlin (1973). It is entirely possible that this enzyme is different from the 49 000-dalton photolyase of Sancar et al. (1983). Fluorescence that could be attributed to a flavin cofactor was not detected in the enzyme samples obtained by ammonium sulfate precipitation or isoelectrofocusing (G. Cimino, personal communication). But it is also clear that this enzyme obtained by the purification procedure described in this work is involved in the photoreactivation process, viz., the repair of the pyrimidine dimer containing DNA by the action of visible light.

The effect of the stimulation of enzyme activity by ATP is not due to the interference of the ATP with the nuclease digestion assay (Sutherland & Chamberlin, 1973) for the following reasons: (1) The concentration of ATP used for the maximum stimulation of enzyme activity was no greater than 2.5 nM, which is about (4×10^2)-fold less than the substrate concentration of the UV-irradiated DNA. This level of ATP concentration caused no interference with the digestion of the substrate by the digest enzymes including alkaline phosphatase. The final concentration of alkaline phosphatase used in the digest reaction mixture was about 0.75 μM, which is the routinely used concentration for 2×10^{-7} M substrate and which is 15-fold greater than the phosphate concentration of the DNA substrate and 300-fold greater than that of ATP. (2) The stimulation of enzyme activity by ATP has been observed by a different assay method (G. Ciarrochi, personal communication) using horizontal agarose gel electrophoresis. The substrate used in this assay was UV-irradiated supercoiled DNA (pAT153). In that experiment, under somewhat different conditions, the Biorex enzyme preincubated with ATP showed a 2-fold stimulation of enzyme activity.

The ATP–enzyme–substrate complex could be formed in any one of the following ways: (1) The enzyme first binds to the substrate, and then the enzyme–substrate complex binds to ATP; (2) the ATP–enzyme complex is formed first and then binds the substrate; or (3) the ATP–enzyme–substrate complex is formed all at once. Following the irradiation of the ATP–enzyme–substrate complex with visible light, photoreactivation might occur by the formation of ADP, inorganic phosphate, and monomerized DNA and the release of the enzyme.

It was recently found for the virgin enzyme that the formation of a stoichiometric complex between the Lys-plasminogen and streptokinase when incubated at 0 °C for 5 min resulted in the formation of an active site in the human Lys-plasminogen (Summaria et al., 1982). Acid dissociation of the complex by streptokinase precipitation retained the active site formed in the plasminogen, and such an activation process was proposed to occur by an intramolecular mechanism. In a similar manner, the requirement for the incubation of the photoreactivating enzyme with ATP to achieve stimulation of enzyme activity could be for the enzyme to assume an active conformation or an active state such as enzyme phosphorylation in the presence of ATP. Such a change of conformation or state from an inactive form that might still bind the substrate to that of an active form could be time dependent after the addition of exogenous ATP. Once the enzyme assumed such an active form, it could retain that form for its recycling process. [^{3}H]ATP can be used to verify protein phosphorylation.

The dissociation of [γ-^{32}P]ATP from the enzyme material bound to UV-irradiation DNA–cellulose (Figure 2) during the time the sample was being exposed to light or kept in the dark is indicative of an equilibrium between the binding of the ATP

to the enzyme or to the enzyme–substrate complex. The time required for the establishment of such an equilibrium could correspond to the time of incubation of the enzyme with ATP to achieve a stimulation of enzyme activity. During the preincubation of the enzyme with ATP, the equilibrium is in favor of the ATP–enzyme complex due to the presence of excess ATP. But once the ATP–enzyme–UV-irradiated DNA–cellulose complex is thoroughly washed with buffer to remove excess unbound ATP, then the ATP could begin to dissociate from the enzyme to establish an equilibrium. Using the yeast enzyme, Harm & Rupert (1968) showed that the enzyme–substrate complexes formed at 37 °C are much more stable at 2 °C. In this work, the ATP–enzyme–substrate complexes were kept at 3 °C either under visible light or in the dark. The observation that detectable enzyme activity was not found in the samples kept in the dark up to 4 h at 3 °C is consistent with the slow rate of dissociation of the enzyme–substrate complex at this temperature. Therefore, there are two types of dissociation from the enzyme material bound to UV-irradiated DNA–cellulose: (1) the dissociation of exogenous ATP, which takes place even in the dark; (2) The dissociation of ATP by hydrolysis into ADP and P_i, which takes place only under light. It is possible that the dialyzable component of the *E. coli* B photoreactivating enzyme (Rupert, 1960) might be ATP.

The reasons for the hydrolysis or utilization of ATP at the time of the photoreactivation process could be varied. (1) It could be utilized for the movement of the enzyme on the UV-irradiated DNA substrate from one pyrimidine dimer to another after the repair of the dimer. (2) It could be utilized for the release of the enzyme from the dimer after its repair. (3) It may actually be involved in the repair of the dimer in conjunction with light. (4) If two ATPs bind to the enzyme, then one ATP could be utilized in moving the enzyme along the substrate and the other could be hydrolyzed during the photoreactivation process. A model for the adenosine binding site on the enzyme protein can be envisaged by testing adenosine-analogue 5′-triphosphates for photoreactivating enzyme activity.

It was found that the photoreceptor phosphodiesterase in the rod outer membrane of vertebrates requires both light and GTP (Bitensky et al., 1978). It was also found that the activation by GTP was 2 times greater than that by ATP. However, this system of light regulation of cyclic GMP levels in rod outer segments consists of several components including proteins. The photoreactivation process is known to consist of the photolyase(s) or the photoreactivating enzyme(s), with additional cofactors or activators depending on the origin or source of the enzyme.

Added in Proof

The G proteins that regulate adenylate cyclase activity can be activated by nonhydrolyzable GTP, and the activation is irreversible (Gilman, 1984). Splicing of eucaryotic mRNA precursors in vitro requires ATP, and the activity of appearance of spliced product is time dependent after the addition of ATP, 45 min to 4 h (Hernandez & Keller, 1983; Hardy et al., 1984; Krainer et al., 1984).

Acknowledgments

I thank Dr. Betsy M. Sutherland for supporting this work and for her advice and suggestions during the course of the experiments. I also thank Dr. John J. Dunn for his keen interest in this work and also for his generous and valuable assistance in providing advice and certain biochemicals (ribonucleases, β,γ-methylene-ATP, and adenovirus genome DNA markers). The high-voltage paper electrophoresis was carried out in Dr. Dunn's laboratory. I thank Drs. B. M. Sutherland, J. J. Dunn, and T. Patterson for critical reading of the manuscript. The preparations of the ammonium sulfate precipitate fraction of the photoreactivating enzyme and the ^{32}P-labeled UV-irradiated T7 DNA substrate by Inan Feng and Alice Shih are greatly appreciated. Many thanks to Louisa Dalessandro for typing the manuscript. I am indebted to the late Dr. Ahmad I. Bukhari (Cold Spring Harbor Laboratory), whose understanding and assistance contributed to the publication of this work.

Registry No. ATP, 56-65-5; GTP, 86-01-1; β,γ-methylene-ATP, 3469-78-1; ADP, 58-64-0; DNA photoreactivating enzyme, 37290-70-3.

References

Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. C., & Ferris, F. L. (1968) *Cold Spring Harbor Symp. Quant. Biol. 33*, 289–305.

Beck, L. A. (1982) Ph.D. Dissertation, University of California, Irvine, CA.

Bitensky, M. W., Wheeler, G. L., Aloni, B., Vetury, S., & Matuo, Y. (1978) *Adv. Cyclic Nucleotide Res. 9*, 553–572.

Boatwright, D. T., Madden, J. J., Denson, J., & Werbin, H. (1975) *Biochemistry 14*, 5418–5423.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Cimino, G., & Sutherland, J. C. (1982) *Biochemistry 21*, 3914–3921.

Cook, J. S. (1967) *Photochem. Photobiol. 6*, 97–100.

Cook, J. S., & Regan, J. D. (1969) *Nature (London) 223*, 1066–1067.

Eker, A., & Ficktinger-Schepman, A. (1975) *Biochim. Biophys. Acta 378*, 54–63.

Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.

Goodgal, S. H., Rupert, C. S., & Herriott, R. M. (1957) in *The Chemical Basis of Heredity* (McElroy, W. D., & Glass, B., Eds.) pp 341–350, Johns Hopkins Press, Baltimore, MD.

Hardy, S. F., Grabowski, P. J., Padgett, R. A., & Sharp, P. A. (1984) *Nature (London) 308*, 375–377.

Harm, H., & Rupert, C. S. (1968) *Mutat. Res. 6*, 355–370.

Hernandez, N., & Keller, W. (1983) *Cell (Cambridge, Mass.) 35*, 89–99.

Koka, P., & Sutherland, B. M. (1980) *Int. Congr. Photobiol., Abstr., 8th*, 316.

Koka, P., & Sutherland, B. M. (1982) *Am. Soc. Photobiol., Abstr., 10th*, 58–59.

Krainer, A. R., Maniatis, T., Ruskin, B., & Green, M. R. (1984) *Cell (Cambridge, Mass.) 36*, 993–1005.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Minato, S., & Werbin, H. (1971) *Biochemistry 10*, 4503–4508.

Richardson, C. C. (1971) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) Vol. 2, pp 815–828, Harper and Row, New York.

Rupert, C. S. (1960) *J. Gen. Physiol. 42*, 573–595.

Rupert, C. S. (1962) *J. Gen. Physiol. 45*, 725–741.

Saito, N., & Werbin, H. (1970) *Biochemistry 9*, 2610–2620.

Sancar, A., & Rupert, C. S. (1978a) *Mutat. Res. 51*, 139–143.

Sancar, A., & Rupert, C. S. (1978b) *Gene 4*, 295–308.

Sancar, G. B., Smith, F. W., & Sancar, A. (1983) *Nucleic Acids Res. 11*, 6667–6678.

Setlow, J. K., & Setlow, R. B. (1963) *Nature (London) 197*, 560–562.

Setlow, J. K., Boling, M. E., & Bollum, F. J. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1430-1436.
Setlow, R. B. (1963) *Science (Washington, D.C.) 153*, 379-386.
Snapka, R. M., & Fuselier, C. (1977) *Photochem. Photobiol. 25*, 415-420.
Snapka, R. M., & Sutherland, B. M. (1980) *Biochemistry 19*, 4201-4208.
Summaria, L., Wohl, R. C., Boreisha, T. G., & Robbins, K. C. (1982) *Biochemistry 21*, 2056-2059.
Sutherland, B. M. (1974) *Nature (London) 248*, 109-112.
Sutherland, B. M. (1981) *Enzymes, 3rd Ed. 14*, 481-515.
Sutherland, B. M., & Chamberlin, M. J. (1973) *Anal. Biochem. 53*, 168-176.
Sutherland, B. M., & Hausrath, S. G. (1979) *J. Bacteriol. 138*, 333-338.
Sutherland, B. M., Court, D., & Chamberlin, M. J. (1972) *Virology 48*, 87-93.
Sutherland, B. M., Chamberlin, M. J., & Sutherland, J. C. (1973) *J. Biol. Chem. 248*, 4200-4205.
Yamamoto, K., Satake, M., Shinagawa, H., & Fujiwara, Y. (1983) *Mol. Gen. Genet. 190*, 511-515.
Youngs, D. A., & Smith, K. C. (1978) *Mutat. Res. 51*, 133-137.

Absence of Swiveling at Sites of Intercalator-Induced Protein-Associated Deoxyribonucleic Acid Strand Breaks in Mammalian Cell Nucleoids†

Yves Pommier,* Michael R. Mattern, Ronald E. Schwartz, and Leonard A. Zwelling

ABSTRACT: The sedimentation of DNA–nuclear protein complexes in 1.9 M salt–neutral sucrose gradients (nucleoid sedimentation) was used to examine the effects of the DNA intercalator 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA) on mouse leukemia cell DNA. Mild detergent cell lysis and neutral pH make nucleoid sedimentation an extremely gentle, but sensitive, method to detect DNA scission. DNA breaks reduce the compaction of nucleoids and slow their sedimentation. Nucleoids from m-AMSA-treated cells sedimented as did those from untreated cells, indicating no detectable m-AMSA-dependent alterations in compaction despite an apparent underlying DNA break frequency of approximately 3 per 10^6 nucleotides, as measured by alkaline elution with proteinase. Mild proteinase digestion of cell lysates prior to nucleoid sedimentation unmasked some, but not all, of the underlying breaks. The frequency of DNA–protein cross-links in nucleoids from cells treated with m-AMSA was comparable to the single-strand break frequency produced by m-AMSA in whole cells. These results indicate that m-AMSA-induced DNA–protein cross-links conceal DNA breaks so as to prevent swiveling around the breaks within the nucleoids. This unique sort of DNA scission is consistent with the involvement of topoisomerases in the DNA breaks elicited by intercalators in mammalian cells.

When mammalian cells are exposed to intercalating agents, DNA strand breaks are produced. These strand breaks are unusual in that they are protein concealed, i.e., their detection by alkaline elution requires proteinase digestion (Ross et al., 1979; Zwelling et al., 1981). Their production appears to be enzymatically mediated (Zwelling et al., 1981) and to depend somewhat upon the three-dimensional structure of the target chromatin (Pommier et al., 1983). For each intercalator break produced, one DNA-protein cross-link is generated, and it has been postulated that the protein is bound covalently to one of the broken DNA strands that it generates (Ross et al., 1979).

In our previous work characterizing this intercalator-induced DNA strand breakage, we utilized two techniques to quantify DNA scission: alkaline sucrose sedimentation and alkaline elution (Zwelling et al., 1981). Both methods employ lysis of cells by alkali and/or detergent prior to quantification of DNA strand breakage. Despite these treatments, DNA–protein binding was detected in the lysates from intercalator-treated cells, suggesting that this binding was covalent. The possibility that the proteins were bound to *both* DNA termini was deemed unlikely because DNA strand breakage was observed by alkaline sedimentation in the absence of proteinase K (Zwelling et al., 1981). However, it was considered possible that both termini could be bound by the enzyme but that one of them was alkali and/or detergent labile, i.e., noncovalent.

DNA topoisomerases are likely candidates for the proteins that are associated with the intercalator-induced strand breaks. We have hypothesized that the protein-associated scission of DNA in cells incubated with intercalators is similar to or identical with the protein-associated DNA strand breakage produced by DNA topoisomerases (Ross et al., 1979; Zwelling et al., 1981). In the case of topoisomerase-induced DNA breaks, it is believed that the enzyme binds to each side of the DNA break it induces, in such a way that only one bond between DNA and each topoisomerase molecule appears to be covalent and that DNA strand movements at the break site are controlled by the enzyme (Cozzarelli, 1979; Gellert, 1981; Pulleyblank & Ellison, 1982). If the protein-associated breaks produced or stabilized by intercalators in mammalian cells or isolated nuclei (Pommier et al., 1982) are produced by topoisomerases, the protein binding should limit movement of the DNA strands around the break sites within chromatin. This possibility has been assessed in the present study by using the nucleoid sedimentation technique.

Nucleoids are DNA-containing and nuclear protein containing structures isolated by the lysis of cells at neutral pH with nonionic detergents and sedimentation in neutral, 1.9 M salt–sucrose gradients (Cook & Brazell, 1975). Histones and most other nonhistone nucleoproteins remain near the top of the gradients while some structural proteins sediment with the

† From the Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received September 1, 1983.*
* Address correspondence to this author at Building 37, Room 5A19.