Binding of *Escherichia coli* DNA Photolyase to UV-Irradiated DNA†

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ABSTRACT: *Escherichia coli* DNA photolyase is a flavoprotein which catalyzes the photomonomerization of pyrimidine dimers produced in DNA by UV irradiation. In vivo, the enzyme acts by a two-step mechanism: it binds to dimer-containing DNA in a light-independent reaction and upon exposure to 300–500-nm light breaks the cyclobutane ring and dissociates from the substrate. Using photolyase purified to homogeneity, we have investigated in vitro the first step of the reaction, DNA binding; enzyme–DNA complex formation was quantitated by the nitrocellulose filter binding assay. We find that the enzyme binds specifically to UV-irradiated DNA regardless of whether the DNA is in the superhelical, open circular, or linear form or whether the DNA is single or double stranded. The binding reaction is optimum at a NaCl concentration of 125 mM and at pH 7.5. Although photolyase is retained by the nitrocellulose filters with near 100% efficiency, the binding efficiency of a single enzyme–substrate complex is about 0.34. The complexes can be dissociated by exposing them to photoreactivating light either in solution or on the filter.

Pyrimidine dimers are the major photoproducts produced in DNA by irradiation at 254 nm [see Patrick & Rahn (1976)] and are the primary lesions responsible for UV-induced mutagenesis and lethality [see Witkin (1976)]. In *Escherichia coli* as well as other organisms, a number of enzymes have evolved which recognize these dimers and remove them from DNA by various mechanisms (Lindahl, 1982; Haseltine, 1983). The specific recognition of dimers by repair enzymes is an intriguing problem; although there is some experimental evidence indicating that dimer formation disrupts hydrogen bonding over a distance of 4 base pairs, thereby creating a distortion in the helix (Kelly et al., 1969; Hayes et al., 1971), recent theoretical calculations suggest that the dimer causes only minimal changes in the helix parameters (Rao et al., 1984). We have recently purified to homogeneity *E. coli* DNA photolyase (EC 4.1.99.3, deoxyribodipyrimidine photolyase) (Sancar et al., 1983; Sancar, A., et al., 1984a). This flavoprotein (Sancar & Sancar, 1984) binds to pyrimidine dimers in DNA and photosensitizes the cleavage of the cyclobutane ring by 300–500-nm light, resulting in situ repiar of dimers. In this paper, we report the results of experiments in which we have used the purified enzyme and the nitrocellulose filter binding assay to determine the optimum conditions for enzyme–substrate complex formation and to analyze the ability of the enzyme to bind to dimers in DNA of various conformations. Our results show that photolyase binds with great specificity to UV-irradiated DNA regardless of whether the dimers are embedded in superhelical, relaxed circular, linear, or single-stranded DNA; this latter result strongly suggests that the enzyme recognizes the dimer, rather than any distortion of the helix. The binding reaction has broad pH and ionic strength optima with peaks at pH 7.5 and μ = 0.125 M, respectively. Photolyase in enzyme–substrate complexes retains full photosensitizing activity when bound to nitrocellulose filters.

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Experimental Procedures

Purification of Photolyase. E. coli DNA photolyase was purified to homogeneity as described previously (Sancar, A., et al., 1984a). The purified enzyme had no endo- or exonucleolytic activity in the absence or presence of Mg^{2+} and/or ATP (data not shown). The purified enzyme was stable for at least 6 months when stored at −80 °C at a concentration >7 mg/mL in a solution containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 50 mM NaCl, 10 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid (EDTA). Protein concentration was determined by the absorbance at 280 nm according to published values for the extinction coefficient of the enzyme (Sancar & Sancar, 1984; Jorns et al., 1984).

Preparation of Radiolabeled Photolyase. E. coli MS09 (Sancar, A., et al., 1984a) was grown to A_{600} = 0.6 in modified Hershey medium (Worcel & Burgi, 1974) supplemented with 1 μg/mL L-methionine, 0.4% glucose, 0.01% threonine and leucine, 0.02% proline and arginine, and 0.0001% thiamin; [^{35}S]methionine (1250 Ci/mmol) and isopropylthio β-D-galactoside (to induce transcription of the *phr* gene which is under the control of the *tac* promoter in the plasmid carried by this strain) were added to final concentrations of 5 μCi/mL and 1 mM, respectively. Incubation was continued at 37 °C for 14 h. The cells were collected by centrifugation, and photolyase was purified as above. From 1 L of culture 5.4 mg of enzyme with a specific activity of 1×10^4 cpm/μg was obtained.

Preparation of Radiolabeled DNA. Plasmid pBR322 DNA was used throughout our experiments. The DNA was labeled with [^{3}H]dT by either of two methods. In the first method, *E. coli* AB2463 (*recA13*)/pBR322 was grown in M9 medium containing 1% casamino acids and 0.0001% thiamin to A_{600} = 0.6 at which time deoxyadenosine, [^{3}H]dT (78.1 Ci/mmol), and chloramphenicol were added to final concentrations of 250 μg/mL, 5 μCi/mL, and 200 μg/mL, respectively. The cells were incubated for 16 h at 37 °C, then collected by centrifugation, and lysed by treatment with lysozyme–Sarkosyl; the plasmid was purified twice by centrifugation in ethidium

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bromide–CsCl gradients (Sancar et al., 1982). DNA prepared by this method had a specific activity of 1.18×10^4 cpm/μg. In the second method, AB2487 (*recA13 thyA*) was grown in M9 medium containing 1% casamino acids, 0.0001% thiamin, and 2.5 μg/mL dT; at $A_{600} = 0.6$, chloramphenicol was added as before. Following 1-h incubation at 37 °C, [^{3}H]dT was added to 2.5 μCi/mL, and incubation was continued for another 16 h. Plasmid DNA was purified as described above and had a specific activity of 3.98×10^4 cpm/μg.

Preparation of UV-Damaged DNA. ^{3}H-Labeled pBR322 DNA containing the desired number of pyrimidine dimers was obtained by irradiating the DNA with 254-nm light from a G.E. germicidal lamp. The DNA was irradiated either in 10 mM Tris (pH 7.4), 10 mM NaCl, and 1 mM EDTA or in various photolyase binding buffers at DNA concentrations ranging from 10 to 100 μg/mL. Because various degrees of shielding were observed under differing irradiation conditions, the average number of pyrimidine dimers per genome was determined for each experiment by the transformation assay of Sancar & Rupert (1978a). Approximately 90% of the lesions induced in DNA by 254-nm radiation are pyrimidine dimers (Patrick & Rahn, 1976). The second major photoproduct is pyrimidine–pyrimidone (6:4) which accounts for about 10% of total photoproducts. We have recently shown that *E. coli* DNA photolyase repairs pyrimidine dimers but not pyrimidine–pyrimidone (6:4) photoproducts (Brash et al., 1984), and therefore, we interpret all our binding data in terms of binding of photolyase to pyrimidine dimers in irradiated DNA as photoproducts other than the two mentioned above constitute only a minor fraction of UV-induced damage.

Filter Binding Assay. Specific reaction conditions for each experiment are described in the figure legends. The general conditions which were constant throughout all experiments were as follows. All reactions were performed under illumination from G.E. "gold" fluorescent bulbs. The enzyme–substrate mixture (102–110 μL) was incubated at 23 °C. At the desired time, 50-μL samples were filtered in duplicate through nitrocellulose filters (Schleicher & Schuell BA85, 24-mm diameter) at a rate of approximately 1 mL/min. All filters had been incubated with reaction buffer overnight at 4 °C and were washed with 1 mL of buffer immediately prior to sample application. After the samples were applied the filters were washed with 3 × 0.3 mL of reaction buffer, dried under a heat lamp, and placed in 7-mL scintillation vials containing 0.5 mL of Cellosolve (Sigma); after the filters had become transparent, 5 mL of ScintiVerse I (Fisher) was added, and the radioactivity was quantitated in an LKB scintillation counter.

For binding experiments with single-stranded DNA, the filters were soaked in 0.1 M KOH for 10 min in a volume of 100 mL, washed with 3 × 100 mL of deionized water, and allowed to soak overnight in reaction buffer at 4 °C. Each filter was washed with 10 mL of the reaction buffer before the sample was applied.

Photoreactivation of Photolyase–DNA Complexes. Enzyme–substrate complexes were exposed, either on nitrocellulose filters or in solution, to light from two Sylvania F15TF8BLB bulbs at a dose rate of 4 J m^{-2} s^{-1} at 360 nm. The amount of ^{3}H-pBR322 DNA retained after filtration and/or washing was determined by liquid scintillation counting. The mean number of remaining enzyme–substrate complexes per molecule was determined as follows. A standard curve of the fraction of molecules retained vs. the mean number of complexes per molecule was constructed by using the Poisson distribution and correcting for the contribution

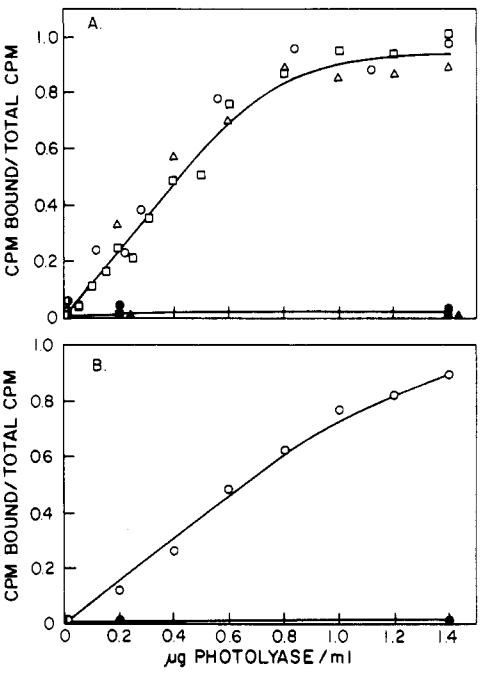

FIGURE 1: (A) Binding of photolyase to DNA in various conformations. ^{3}H-pBR322 DNA was incubated at a concentration of 4 μg/mL with photolyase at the indicated concentrations; incubation was for 1 h in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 100 μg/mL bovine serum albumin. (○, ●) Covalently closed circular DNA; (□, ■) open circular DNA; (△, ▲) linear DNA produced by cleavage of covalently closed circular DNA with *Eco*RI followed by phenol extraction. Open symbols, UV-irradiated DNA containing an average of 10 dimers per genome; closed symbols, nonirradiated DNA. (B) Binding of photolyase to single-stranded DNA. ^{3}H-pBR322 DNA (specific activity 39 000 cpm/μg) was linearlized with *Eco*RI, extracted with phenol, irradiated to give an average of eight dimers per genome, then denatured by boiling for 10 min, and quickly on cooled ice. The DNA was incubated at a concentration of 4.2 μg/mL with increasing concentrations of photolyase as described in Figure 2. (○) Irradiated DNA; (●) nonirradiated DNA.

of the P_1 and P_2 classes for a retention efficiency of 0.34 and 0.68, respectively (see Results). The mean number of complexes per molecule for individual data points was determined from this curve. The fraction of complexes removed ($[ES]_R$) at time t was then calculated according to the formula

$$[ES]_R = \frac{[ES]_0 - [ES]_t}{[ES]_0}$$

where $[ES]_0$ and $[ES]_t$ are the number of complexes present at time 0 and time t, respectively.

RESULTS

Photolyase Binds to UV-Irradiated DNA Specifically. In Figure 1, the binding in vitro of the purified enzyme to irradiated and nonirradiated DNA is compared; the enzyme binds much more efficiently to irradiated DNA. At an enzyme concentration of 0.84 μg/mL, at which 90% of the irradiated DNA is retained on the filters, there is no detectable binding (<1%) to nonirradiated DNA. Only at the highest enzyme concentration used is some nonspecific binding detected, amounting to about 2% of the binding to irradiated DNA.

In the initial portion of the binding curve shown in Figure 1, dimers are in molar excess compared to photolyase, and thus, enzyme–substrate complex formation is first order with respect to photolyase. Using the standard curve described under Experimental Procedures, it can be shown that at about ≤50%

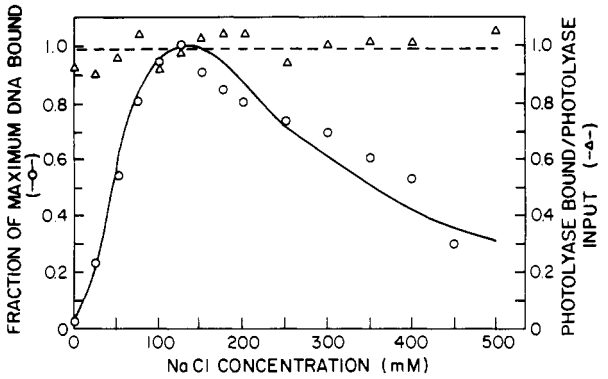

FIGURE 2: Effect of NaCl concentration on binding of photolyase to UV-irradiated DNA and to nitrocellulose filters. ^{3}H-pBR322 DNA (11 800 cpm/μg) containing an average of 10 dimers/genome was incubated with photolyase in a reaction mixture consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM β-mercaptoethanol, 100 μg/mL bovine serum albumin, and NaCl at the indicated concentrations; the concentrations of DNA and photolyase were 3.3 and 0.2 μg/mL, respectively. Binding of photolyase–DNA complexes (O) is given as the fraction of the maximum binding observed. In separate experiments, ^{35}S-labeled photolyase (0.15 or 10 μg/mL; 10 200 cpm/μg) was incubated in reaction buffer plus NaCl but without DNA and then filtered. Photolyase bound (Δ) is expressed as a fraction of the total amount of photolyase applied to the filter.

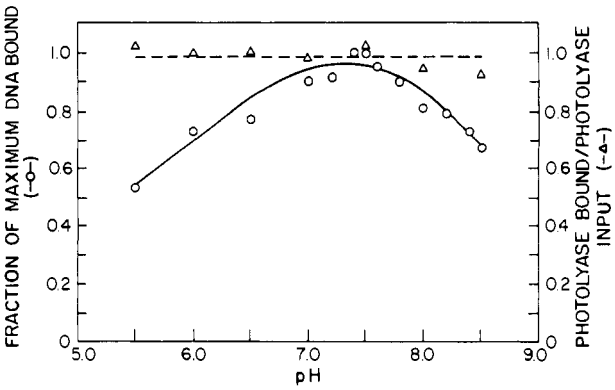

FIGURE 3: Effect of pH on binding of photolyase to UV-irradiated DNA and to nitrocellulose filters. ^{3}H-pBR322 DNA (specific activity 11 800 cpm/μg) containing an average of 10 dimers/genome was incubated at a concentration of 3.3 μg/mL with photolyase at 0.2 or 0.1 μg/mL in a reaction mixture consisting of 50 mM potassium phosphate at the indicated pH, 1 mM EDTA, 1 mM β-mercaptoethanol, and 100 μg/mL bovine serum albumin. Binding of photolyase–DNA complexes (O) is given as the fraction of the maximum binding observed. In separate experiments, ^{35}S-labeled photolyase (20 μg/mL; 10 200 cpm/μg) was incubated in reaction buffer minus DNA and then filtered. Photolyase bound (Δ) is expressed as a fraction of the total amount of photolyase applied to the filter.

of total counts bound the number of molecules retained on the filter equals the mean number of complexes present in solution divided by the efficiency of retention of a single complex (see below); therefore, binding of ES complexes to the filters is also first order in this region. Extrapolation of the initial slope in Figure 1 to 100% retention (i.e., 10 complexes per molecule on average) yields the point at which dimers and enzyme are present at equimolar concentrations; since the concentration of dimers is accurately known, this value provides a measure of the number of photolyase molecules able to bind DNA. We find that 0.8 μg or 14.8 pmol of photolyase (Sancar, G. B., et al., 1984) is capable of binding 13.9 pmol of dimers and from this conclude that at least 94% of photolyase molecules are active in DNA binding.

Binding of Photolyase to Nitrocellulose Filters. Studies on DNA–protein interactions using nitrocellulose filters are based on the observation that while proteins are retained by the filters double-stranded DNA is not unless it is complexed with a protein (Riggs et al., 1968). However, it is known that binding of proteins to nitrocellulose filters is not necessarily 100% efficient under all experimental conditions (Yarus & Berg, 1967, 1970; Riggs et al., 1970a; Hinkle & Chamberlin, 1972; Strauss et al., 1981). Since binding efficiency is an important parameter in interpreting DNA–protein interactions as measured by the filter binding assay, we determined the amount of photolyase bound to nitrocellulose filters using ^{35}S-labeled protein. The results (data not shown) indicate that under standard assay conditions photolyase is quantitatively retained by the filters over a range of 0.7–10^4 ng of enzyme per filter.

Effect of Ionic Strength on Photolyase–UV-Irradiated DNA Binding. Figure 2 shows the effect of NaCl concentration on the formation of photolyase–UV-irradiated DNA complexes. The reaction is optimum at 125 mM NaCl; however, significant binding is observed at higher ionic strength such that at 500 mM NaCl there is still 30% of maximum binding. That these results reflect enzyme–substrate complex formation and not simply a change in the efficiency of retention of the enzyme by the filter is supported by the fact that the enzyme is retained quantitatively over the entire range of NaCl concentrations used in this experiment (Figure 2).

Effect of pH on Enzyme–Substrate Complex Formation. The effect of pH on binding of photolyase to UV-irradiated DNA is shown in Figure 3. A broad optimum between pH 7 and 7.6 is observed, and even at the extremes of pH used in this experiment, pH 5.5 and 8.5, there is still significant binding (50% and 65% of the maximum, respectively). As in the experiments on the effect of ionic strength, there is very little change in the retention of photolyase as a function of pH (Figure 3).

Effect of DNA Conformation on Complex Formation. Having determined the optimum conditions for binding of photolyase to UV-irradiated DNA, we next asked whether the conformation of the substrate affects enzyme–substrate complex formation. As can be seen in Figure 1A, the enzyme binds equally well to superhelical, relaxed circular, and linear DNA. The results suggest that the enzyme upon binding does not unwind the DNA as it is known that molecules that unwind DNA bind more tightly to superhelical DNA than to linear or open circular forms (Bauer & Vinograd, 1968, 1970; Maniatis & Ptashne, 1973; Wang et al., 1974).

Binding of Photolyase to Single-Stranded DNA. DNA binding proteins that recognize specific sequences (e.g., RNA polymerase and *lac* and λ repressor) bind specifically only to double-stranded DNA. However, photolyase recognizes pyrimidine dimers and therefore might be expected to bind specifically to UV-irradiated DNA in both single- and double-stranded form. Figure 1B shows that the enzyme does indeed show the same specificity for UV-irradiated single-stranded DNA as for double-stranded DNA. Comparison of the data for binding of single-stranded vs. double-stranded substrate shows that at the same enzyme and DNA concentrations the fraction of single-stranded DNA retained on the filters is half of that seen with double-stranded DNA. This is not because of a lower affinity of the enzyme for dimers in single-stranded DNA; rather, it is due to the fact that each enzyme–single-stranded DNA complex results in retention of only half the mass of DNA present in an enzyme–double-stranded DNA complex.

Effect of Dimer Concentration on Retention of Photo-

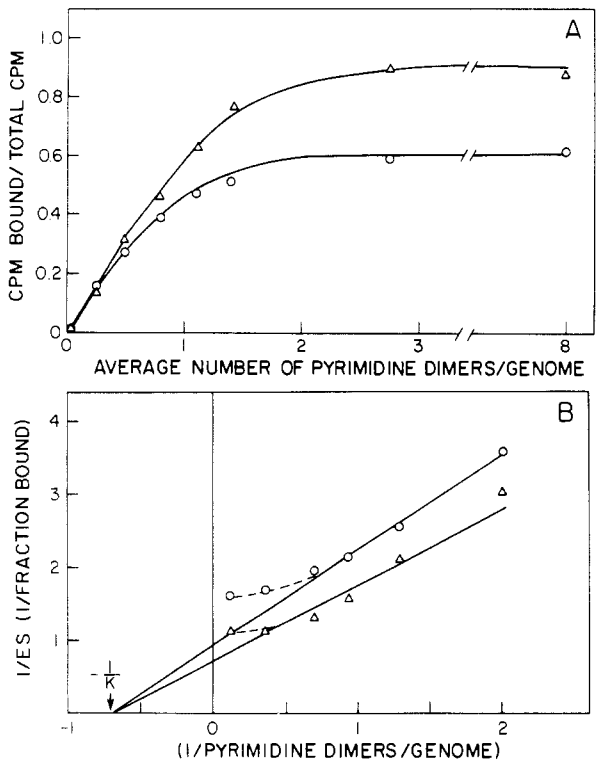

FIGURE 4: (A) Binding of photolyase to DNA as a function of the number of pyrimidine dimers per genome. ^{3}H-pBR322 DNA (specific activity 39 000 cpm/μg), irradiated to give the number of dimers indicated, was incubated at a concentration of 34 μg/mL with either 2.4 (○) or 5.6 μg/mL (△) photolyase in reaction buffer as described in Figure 1. (B) Double-reciprocal plot of $1/[ES]$ vs. $1/S$ where [ES] is the cpm bound per total cpm and S is the average number of pyrimidine dimers per genome. Data for this plot are taken from the binding isotherms shown in panel A, and the symbols are the same. The intercept with the x axis is -0.69.

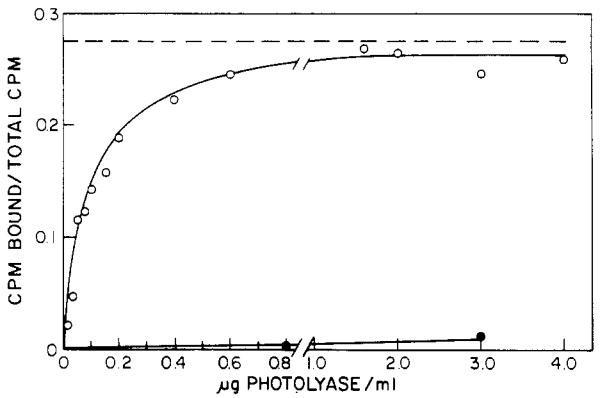

FIGURE 5: Binding of photolyase to DNA containing an average of 0.85 dimer/genome. ^{3}H-pBR322 DNA at 3.2 μg/mL was incubated with increasing concentrations of photolyase in the reaction buffer described in Figure 1. (○) pBR322 DNA containing an average of 0.85 dimer/molecule; (●) nonirradiated DNA; (dashed line) expected saturation value for a photolyase–DNA complex binding efficiency of 0.34. The maximum binding observed with nonirradiated DNA was 1% of the input counts.

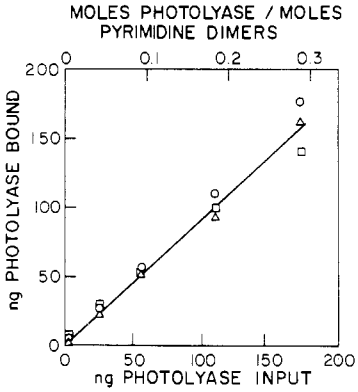

FIGURE 6: Binding of photolyase to nitrocellulose filters in the presence or absence of a molar excess of pyrimidine dimers. Increasing concentrations of ^{35}S-labeled photolyase were incubated in reaction buffer as described in Figure 1; reaction mixtures contained either no DNA (□), UV-irradiated DNA (○) (89 μg/mL, 13 dimers/pBR322 genome), or nonirradiated DNA (△) (89 μg/mL).

lyase–DNA Complexes on the Filters. The binding experiments described in the previous sections were carried out with DNA that contained greater than or equal to eight dimers per double-stranded molecule. When the number of dimers per molecule was chosen as the variable at given DNA and protein concentrations, the results shown in Figure 4A were obtained. When these binding isotherms were analyzed by the double-reciprocal plot of Edsall & Wyman (1958) (Figure 4B), we obtained an intercept at the x axis of -0.69; this value is the negative reciprocal of the number of enzyme–substrate complexes per molecule required for 50% of the molecules to be bound to the filter which therefore equals 1.45. Thus, 2.90 complexes per molecule are required for each pBR322 genome to be retained with 100% efficiency; the probability of retention of a molecule with one enzyme–substrate complex is thus $1/2.90 = 0.34$. The same conclusion was reached with a different experiment in which pBR322 DNA containing an average of 0.85 dimers/genome was used as substrate in the filter binding assay. According to the Poisson distribution, 43% of the molecules will not contain dimers, and if every enzyme–substrate complex was retained by the filter, 57% of the total molecules should be retained at saturating amounts of enzyme. However, the results shown in Figure 5 demonstrate that the maximum retention obtained is only 26.5%. This value agrees quite closely with the 27.5% retention calculated by assuming a Poisson distribution of dimers and a retention efficiency of 0.34 for each enzyme–substrate complex.

Effect of DNA on Retention of Photolyase by Filters. Since photolyase alone is retained efficiently by nitrocellulose filters, the low efficiency of retention of enzyme–substrate complexes could be due to either of two factors: either DNA-bound protein is somehow protected against binding to the filter or the filter-bound complexes dissociate on the filter and the released DNA passes through (Woodbury & von Hippel, 1983). To differentiate between the possibilities, radiolabeled photolyase was incubated with DNA containing a molar excess of dimers over photolyase, and after time was allowed for complete complex formation, the mixture was filtered. As is seen in Figure 6, photolyase is quantitatively retained at all concentrations, leading us to conclude that the low efficiency of complex retention is due to the dissociation of DNA and enzyme on the filter.

Release of Filter-Bound DNA by Photoreactivating Light. The fact that DNA binding proteins remained complexed to their substrates during filtration indicates that these proteins retain at least some of their "native" conformation. It has been suggested, however, that partial unfolding of the proteins may contribute to their retention (Woodbury & von Hippel, 1983). Thus, it was of interest to determine whether filter-bound photolyase could carry out the second step of its enzymic reaction, photolysis. A reaction mixture containing photolyase–UV-irradiated DNA complexes was filtered through nitrocellulose; the filters were exposed to photoreactivating light for various lengths of time and washed with reaction buffer,

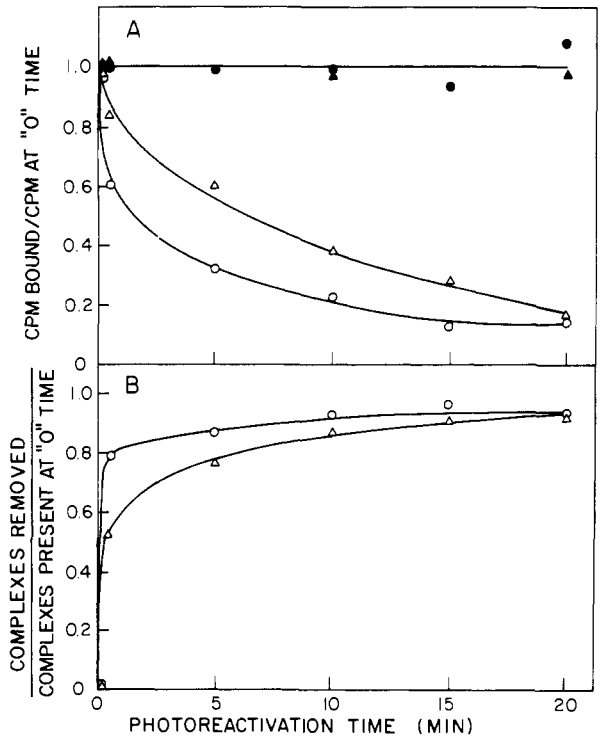

FIGURE 7: Dissociation of photolyase–DNA complexes by photoreactivating light. (A) ^{3}H-pBR322 DNA containing an average of eight dimers per genome was incubated at 4.2 μg/mL with photolyase at a concentration of 5.4 μg/mL in the reaction buffer described in Figure 2. Following isolation of photolyase–DNA complexes on nitrocellulose filters, the complexes were either exposed to photoreactivating light (○) or kept in the dark (●) for the indicated time and then washed once with reaction buffer, and the remaining counts were determined. In a separate experiment, ^{3}H-pBR322 DNA (eight dimers per genome) was incubated at a concentration of 3.4 μg/mL with photolyase at 6.4 μg/mL. One hour after addition of enzyme, the reaction mix was divided into two parts, one of which was exposed to photoreactivating light (△) while the other was kept in the dark (▲). Aliquots of 50 μL were taken at the indicated times and filtered. (B) The data from panel A were used to determine the average number of photolyase–DNA complexes dissociated by photoreactivating light, as described under Experimental Procedures. Symbols are as described in panel A. The mean number of complexes per molecule at zero time was taken to be 7.2, on the basis of our observation (unpublished) that at these enzyme and substrate concentrations 90% of dimers can be repaired by four sequential intense light flashes over a 3-s period.

and the amount of DNA remaining on the filter was measured as a function of illumination time. As can be seen in Figure 7A, illumination results in a sharp decrease in the amount of DNA retained, indicating that filter-bound photolyase is capable of photolysis. Similar kinetics were obtained when the enzyme–substrate complexes were photoreactivated before filtration, leading us to conclude that the filter-bound enzyme is in a form close to that of the native enzyme in solution. (The values for the number of complexes removed in Figure 7B is a minimum estimate in the case of solution photoreactivation because throughout photoreactivation new complexes are free to form.)

Discussion

The results reported in this paper lead to the following conclusions concerning *E. coli* DNA photolyase. (1) The enzyme binds specifically to DNA containing pyrimidine dimers. (2) At the macromolecular level, photolyase is insensitve to the conformation of the substrate, binding equally well to superhelical circular, relaxed circular, linear, and single-stranded UV-irradiated DNA. (3) Photolyase binds quantitatively to nitrocellulose filters over a wide range of pHs and NaCl concentrations; however, binding of photolyase–UV-irradiated DNA complexes is optimal at pH 7.4–7.6 and 125 mM NaCl. (4) The binding efficiency of photolyase–UV-irradiated DNA complexes to nitrocellulose filters is low; under our standard assay conditions, each complex has a probability of 0.34 of binding to the filter. (5) Filter-bound photolyase is capable of performing the second step of its reaction, photolysis. The implications of these findings for the specific problem of photolyase–UV-irradiated DNA binding and the general area of DNA–protein interactions as assayed by the nitrocellulose filter binding technique are discussed below.

At subsaturating enzyme concentrations, photolyase binds UV-irradiated DNA at least 100 times more efficiently than it binds nonirradiated DNA. In this respect, the enzyme is similar to several other enzymes involved in the repair of pyrimidine dimers, namely, *S. cerevisiae* photolyase (Madden et al., 1973), phage T4 endonuclease V (Seawell et al., 1980), and *Micrococcus luteus* pyrimidine dimer glycosylase–apyrimidinic endonuclease (Riazuddin & Grossman, 1977), but is markedly different from *E. coli* UvrABC excision nuclease (Sancar & Rupp, 1983) which also acts on pyrimidine dimers. The UvrA and UvrB subunits of the latter enzyme have been implicated in dimer recognition; however, at subsaturating concentrations, UvrA binds only 3–5 times more efficiently to dimer-containing DNA than to nonirradiated DNA (Kacinski et al., 1981; Seeburg & Steinum, 1982), and addition of UvrB improves this discrimination by no more than a factor of 3 (Kacinski & Rupp, 1981; Yeung et al., 1983). The difference in binding specificity of photolyase vs. UvrABC excision nuclease presumably reflects the fact that the former enzyme acts only on cis-syn pyrimidine dimers (Ben-Hur & Ben-Ishai, 1968) while the latter enzyme recognizes as damage a number of structurally dissimilar DNA adducts [for a review, see Witkin (1976)] and thus may sacrifice some specificity for versatility. In this context, it is interesting to note that in vivo Phr$^+$Uvr$^+$ cells are more resistant to UV killing in the absence of photoreactivation than are Phr$^-$Uvr$^+$ cells (Harm & Hillebrandt, 1962; Yamamoto et al., 1984), and we have recently demonstrated in vitro that photolyase under non-photoreactivating conditions stimulates both the rate and extent of cutting by UvrABC excision nuclease of UV-irradiated DNA specifically (Sancar, A., et al., 1984b). The cutting sites of the excision nuclease are situated on a single face of the double helix (Sancar & Rupp, 1983); thus, it is tempting to speculate that binding of photolyase to another face of the helix increases the ability of the nuclease to discriminate between damaged and undamaged DNA.

The secondary and higher order structure of UV-irradiated DNA apparently has no effect on the binding of photolyase to pyrimidine dimers. It has been shown both theoretically (Bauer & Vinograd, 1968, 1970; Davidson, 1972) and experimentally (Maniatis & Ptashne, 1973; Wang et al., 1974) that molecules which induce unwinding in DNA bind preferentially to negatively supercoiled (partially unwound) DNA; the fact that photolyase binds equally well to supercoiled and relaxed circular DNA indicates that the enzyme does not unwind the region around the dimer to any significant extent if at all. It is also significant that photolyase shows the same affinity for single-stranded and double-stranded dimer-containing DNA as this is a strong indication that the dimer is the entity recognized by the enzyme rather than distortion caused by the dimer in double-stranded DNA. This conclusion is in agreement with in vitro studies showing that single- and double-stranded φX174 DNAs are equally good substrates for

photoreactivation (Mennigmann, 1975) and with in vitro studies demonstrating that the rate of the enzymic reaction is the same for single- and double-stranded DNA containing pyrimidine dimers (Sancar, A., et al., 1984a; Jorns et al., 1985).

As measured by the nitrocellulose filter binding assay, optimum conditions for the binding of *E. coli* photolyase to irradiated DNA are pH 7.4–7.6 and an ionic strength of μ = 0.125–0.150. While these conditions are similar to the optimum conditions reported for photolyase from *S. cerevisiae* (Madden & Werbin, 1974), the behavior of the two enzymes over the range of pHs and ionic strengths tested is quite different. Thus, binding of DNA by yeast photolyase exhibits a sharp maximum at pH 7.6–7.8, and less than 25% binding is seen below pH 6.5, while binding by *E. coli* photolyase is much less pH dependent, and 55% of maximum binding is still observed at pH 5.5. Binding is maximal with yeast photolyase at μ = 0.175, and no binding is detectable at $\mu > 0.4$ whereas with the *E. coli* enzyme 30% of maximum binding is found at μ = 0.5. The dependence of binding cosntants upon ionic strength has been interpreted in the case of DNA binding proteins in terms of ion pair formation between phosphate groups on DNA and positively charged groups on the protein. As it is known that yeast photolyase requires a minimum substrate length of nine nucleotides (Setlow & Bollum, 1968) while *E. coli* photolyase will catalyze photomonomerization on substrates as small as $(dT)_3$ (Jorns et al., 19845), it is reasonable to assume that the differential effect of salt on the enzymes is due to a greater number of ion pairs being required for binding of yeast photolyase than for the *E. coli* enzyme. To substantiate this speculation, it will be necessary to determine the K_{assoc} for photolyase at the various salt concentrations used in our experiments and thereby directly determine the number of ion pairs formed at the DNA–protein interface during binding. It is interesting to note that phage T4 endonuclease V, which also binds to pyrimidine dimers, shows optimum specificity for irradiated DNA at 2.1 M NaCl/0.21 M trisodium citrate (Seawell et al., 1980); thus, it appears that for *E. coli* photolyase and T4 endonuclease V nonionic interactions are important for binding to DNA.

The variation in binding of photolyase–DNA complexes to nitrocellulose as a function of pH and ionic strength is in marked contrast to the binding of photolyase alone to the filters; the enzyme is quantitatively retained over the entire range of pHs and ionic strengths tested and up to at least 10 μg of photolyase per 24-mm-diameter filter. Furthermore, formation of enzyme–substrate complexes does not affect the binding of photolyase to the filters even under conditions in which essentially all photolyase molecules are complexed with DNA. It is these observations which make possible our conclusion that the measured retention of DNA on the filter reflects enzyme–substrate interactions in solution. Although the exact mechanism for attachment of proteins to nitrocellulose filters is not known, hydrogen bonding and ionic and hydrophobic interactions are all thought to be contributing factors (Woodbury & von Hippel, 1983). The fact that *E. coli* RNA polymerase is quantitatively retained over salt concentrations ranging from 50 to 150 mM KCl has been interpreted as indicating that ionic interactions are not important for filter binding (Strauss et al., 1981). The results we have obtained with *E. coli* DNA photolyase support this conclusion and suggest that hydrophobic interactions are the main determinants in protein–nitrocellulose filter binding. However, it should be pointed out that retention of RNA polymerase increases linearly with salt concentration between 0 and 50 mM KCl while photolyase is quantitatively retained in 10 mM Tris in the absence of any additional salt. Thus, at low ionic strength, the mechanism of binding of these proteins to filters may differ.

Our results indicate that the binding efficiency of a single photolyase–DNA complex is 0.34. The efficiency of binding of varius protein–DNA complexes has been reported to range from 1.0 for *Eco*RI endonuclease (Terry et al., 1983) to 0.4 for the *lac* repressor (Riggs et al., 1970a), 0.2 for yeast photolyase (Madden et al., 1973), 0.1 for phage T4 endonuclease V (Seawell et al., 1980), and 0 for *lexA* protein (Dr. Roger Brent, personal communication). It has been suggested that inefficient retention results from the nonequilibrium nature of the filter assay (Woodbury & von Hippel, 1983), that is, that the bound complexes dissociate during filtration, and the protein cannot recapture the DNA. While this may in part account for variations in retention efficiencies, the fact that the *lac* repressor and *Eco*RI endonuclease have similar dissociation rate constants [$k_{off} \approx 6.5 \times 10^{-4}$ s^{-1} (Riggs et al., 1970b; Jack et al., 1982)] but very different retention efficiencies argues that additional factors must be important. One likely explanation is that upon binding to the filter proteins undergo a conformational change which affects k_{off} to various degrees for each protein. This model does not necessarily imply that such a conformational change results in complete denaturation of the protein as yeast photolyase (Sancar & Rupert, 1978b), polynucleotide phosphorylase (Thang et al., 1968), and *lac* repressor (Gilbert & Maxam, 1973) retain at least partial activity when bound to nitrocellulose filters. In this context, it is interesting to note that both the extent of photoreactivation which we observe with filter-bound photolyase and the similarity of the observed kinetics to those obtained by Harm (1970) in vivo suggest that the filter-bound enzyme is fully active with respect to photolysis.

Registry No. DNA photolyase, 37290-70-3.

References

Bauer, W., & Vinograd, J. (1968) *J. Mol. Biol. 33*, 141–171.

Bauer, W., & Vinograd, J. (1970) *J. Mol. Biol. 47*, 419–435.

Ben-Hur, E., & Ben-Ishai, R. (1968) *Biochim. Biophys. Acta 166*, 9–15.

Brash, D. E., Franklin, W. A., Sancar, G. B., Sancar, A., & Haseltine, W. A. (1984) *J. Biol. Chem.* (submitted for publication).

Davidson, N. (1972) *J. Mol. Biol. 66*, 307–309.

Edsall, J. T., & Wyman, J. (1958) in *Biophysical Chemistry*, pp 610–613, Academic Press, New York.

Gilbert, W., & Maxam, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3581–3584.

Harm, W. (1970) *Mutat. Res. 10*, 277–290.

Harm, W., & Hillebrandt, B. (1962) *Photochem. Photobiol. 1*, 271–272.

Haseltine, W. A. (1983) *Cell (Cambridge, Mass.) 33*, 13–17.

Hayes, F. N., Williams, D. L., Ratliffe, R. L., Varghese, A. J., & Rupert, C. S. (1971) *J. Am. Chem. Soc. 93*, 4940–4942.

Hinkle, D. C., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157–185.

Jack, W. E., Terry, B. J., & Modrich, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4010–4014.

Jorns, M. S., Sancar, G. B., & Sancar, A. (1984) *Biochemistry 23*, 2673–2679.

Jorns, M. S., Sancar, G. B., & Sancar, A. (1985) *Biochemistry* (following paper in this issue).

Kacinski, B. M., & Rupp, W. D. (1981) *Nature (London) 294*, 480–481.
Kacinski, B. M., Sancar, A., & Rupp, W. D. (1981) *Nucleic Acids Res. 9*, 4495–4508.
Kelly, R. B., Atkinson, M. R., Huberman, J. A., & Kornberg, A. (1969) *Nature (London) 224*, 495–501.
Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61–87.
Madden, J. J., & Werbin, H. (1974) *Biochemistry 13*, 2149–2154.
Madden, J. J., Werbin, H., & Denson, J. (1973) *Photochem. Photobiol. 18*, 441–445.
Maniatis, T., & Ptashne, M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1531–1535.
Mennigmann, H.-D. (1975) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 27*, 313–323.
Patrick, M. H., & Rahn, R. O. (1976) *Photochem. Photobiol. Nucleic Acids 2*, 35–95.
Rao, S. N., Keepers, J. W., & Kollman, P. (1984) *Nucleic Acids Res. 12*, 4789–4807.
Riazuddin, S., & Grossman, L. (1977) *J. Biol. Chem. 252*, 6280–6286.
Riggs, A. D., Bourgeois, S., Newby, R. F., & Cohn, M. (1968) *J. Mol. Biol. 34*, 365–368.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970a) *J. Mol. Biol. 48*, 67–83.
Riggs, A. D., Bourgeois, S., & Cohn, M. (1970b) *J. Mol. Biol. 53*, 401–417.
Sancar, A., & Rupert, C. S. (1978a) *Nature (London) 272*, 471–472.
Sancar, A., & Rupert, C. S. (1978b) *Gene 4*, 295–308.
Sancar, A., & Rupp, W. D. (1983) *Cell (Cambridge, Mass.) 33*, 249–260.
Sancar, A., & Sancar, G. B. (1984) *J. Mol. Biol. 172*, 223–227.
Sancar, A., Smith, F. W., & Sancar, G. B. (1984a) *J. Biol. Chem. 259*, 6028–6032.
Sancar, A., Franklin, K. A., & Sancar, G. B. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7397–7401.
Sancar, G. B., Sancar, A., Little, J. W., & Rupp, W. D. (1982) *Cell (Cambridge, Mass.) 28*, 523–530.
Sancar, G. B., Smith, F. W., & Sancar, A. (1983) *Nucleic Acids Res. 11*, 6667–6678.
Sancar, G. B., Smith, F. W., Lorence, M. C., Rupert, C. S., & Sancar, A. (1984) *J. Biol. Chem. 259*, 6033–6038.
Seawell, P. C., Simon, T. J., & Ganesan, A. K. (1980) *Biochemistry 19*, 1685–1691.
Seeberg, E., & Steinum, A.-L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 988–992.
Setlow, J. K., & Bollum, F. J. (1968) *Biochim. Biophys. Acta 157*, 233–237.
Strauss, H. S., Boston, R. S., Record, M. T., Jr., & Burgess, R. R. (1981) *Gene 13*, 75–87.
Terry, B. J., Jack, W. E., Rubin, R. A., & Modrich, P. (1983) *J. Biol. Chem. 258*, 9820–9825.
Thang, M. N., Graffe, M., & Grunberg-Menago, M. (1968) *Biochem. Biophys. Res. Commun. 31*, 1–8.
Wang, J. C., Barkley, M. D., & Bourgeois, S. (1974) *Nature (London) 251*, 247–249.
Witkin, E. M. (1976) *Bacteriol. Rev. 40*, 869–907.
Woodbury, C. P., Jr., & von Hippel, P. H. (1983) *Biochemistry 22*, 4730–4737.
Worcel, A., & Burgi, E. (1974) *J. Mol. Biol. 82*, 91–105.
Yamamoto, K., Satake, M., & Shinagawa, H. (1984) *Mutat. Res. 131*, 11–18.
Yarus, M., & Berg, P. (1970) *Anal. Biochem. 35*, 450–465.
Yeung, A. T., Mattes, W. B., Oh, E. Y., & Rossman, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6157–6161.