Action Mechanism of *Escherichia coli* DNA Photolyase

I. FORMATION OF THE ENZYME-SUBSTRATE COMPLEX*

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*Escherichia coli* DNA photolyase (photoreactivating enzyme) is a flavoprotein. The enzyme binds to DNA containing pyrimidine dimers in a light-independent step and, upon illumination with 300–600 nm radiation, catalyzes the photosensitized cleavage of the cyclobutane ring thus restoring the integrity of the DNA. We have studied the binding reaction using the techniques of nitrocellulose filter binding and flash photolysis. The enzyme binds to dimer-containing DNA with an association rate constant $k_1$, estimated by two different methods to be $1.4 \times 10^9$ to $4.2 \times 10^9$ M$^{-1}$ s$^{-1}$. The dissociation of the enzyme from dimer-containing DNA displays biphasic kinetics; for the rapidly dissociating class of complexes $k_2 = 2-3 \times 10^{-2}$ s$^{-1}$, while for the more slowly dissociating class $k_2 = 1.3 \times 10^{-2}$ to $6 \times 10^{-2}$ s$^{-1}$. The equilibrium association constant $K_4$, as determined by the nitrocellulose filter binding assay and the flash photolysis assay, was $4.7 \times 10^6$ to $6 \times 10^6$ M$^{-1}$, in reasonable agreement with the values predicted from $k_1$ and $k_2$. From the dependence of the association constant on ionic strength we conclude that the enzyme contacts no more than two phosphodiester bonds upon binding; this strongly suggests that the pyrimidine dimer is the main structural determinant of specific photolyase-DNA interaction and that nonspecific ionic interactions do not contribute significantly to substrate binding.

Irradiation of DNA with 254 nm of light induces the formation of dimers between adjacent pyrimidine bases in the same DNA strand; left unrepaird such lesions result in mutation or cell death. DNA photolyases (EC 4.1.99.3) are a group of enzymes which catalyze the light-dependent reversal of pyrimidine dimers in DNA thus repairing these lesions in *situ*. The reaction proceeds by a two-step mechanism (Rupert, 1962): (i) the substrate binding step, which can occur in the dark, and (ii) dimer reversal, which occurs only in the presence of light, the precise wavelength being dependent upon the source of the enzyme (see Harm, 1976, for a review). Thus the overall reaction scheme can be written as the following.

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P$$

Although the first evidence for the enzymatic nature of photoreactivation was obtained using crude extracts from

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solutions (5–10 mg/ml) retained this color for over a year when stored at −80 °C. We will refer to such enzyme as “blue enzyme.” In contrast to the stability of the enzyme under the conditions described above, prolonged purification procedures and/or storage at −20 °C in 40% ethylene glycol containing 10 mM β-mercaptoethanol for more than 6 months results in the appearance of “yellow enzyme.” Spectrophotometric titration at 450 nm indicates that 70% of the FAD in yellow enzyme was in the fully oxidized form (Jorns et al., 1987). The oxidized FAD remained firmly associated with the enzyme as overnight dialysis of the yellow enzyme did not result in a detectable decrease in the 450-nm absorption.

Visualization of ES Complexes by Electron Microscopy—DNA molecules that contained pyrimidine dimers in a slightly off-center location were prepared as follows. Plasmid pBR322 DNA was digested with EcoRI and BamHI restriction endonucleases and the (EcoRI-BamHI)ss and (BamHI-EcoRI)ss fragments were separated on agarose gels and purified as described by Vogelstein and Gillespie (1979). The small fragment was irradiated with 1.5 kJ/m² of 254 nm of light to produce an average of 3 pyrimidine dimers/molecule. The irradiated DNA was mixed with the nonirradiated (BamHI-EcoRI)ss fragment at a concentration of 4 µg/ml each and covalently linked using T4 DNA ligase. After overnight incubation at 14 °C, ligase was inactivated by heating at 65 °C for 10 min. The DNA was then digested with PvuII (which cuts at base pair 2067) and separated on a 1% agarose gel; a full-length fragment (4563 base pairs) was purified from this gel and used for electron microscopic studies. Photolyase-DNA complexes were fixed with 1% formaldehyde, 0.6% glutaraldehyde, mounted onto thin carbon films, and rotary shadowed with tungsten (Griffith and Christiansen, 1978); micrographs were taken using a Phillips EM400 TLG electron microscope.

Preparation of Substrate for Filter Binding and Flash Photolysis Studies—Radiolabeled pBR322 DNA was obtained from E. coli AB2463 (recA13)/pBR322 as previously described (G. Sancar et al., 1984a, 1984b; Sancar and Rupert, 1978a; A. Sancar et al., 1985). The specific activity was 1.3 × 10⁹ cpm/µg. The DNA was diluted to a concentration of 20 µg/ml and irradiated with 254 nm of light. The average number of UV-induced lethal lesions/molecule was determined by transformation selecting for tetracycline resistance (Sancar and Rupert, 1978a; A. Sancar et al., 1984a, 1984b; G. Sancar et al., 1986); incubation of UV-irradiated DNA with a molar excess of photolyase for 1 h under photoreactivating light (supplied by a Sylvania F15T8SBL bulb at a fluence rate of approximately 10 J/m²), followed by transformation was used to quantitate the average number of UV-induced pyrimidine dimers/molecule. When performed as described, the transformation assay is a sensitive and accurate method for quantitating dimmer repair. Typically transformation with 0.1 µg of plasmid DNA containing an average of 6 pyrimidine dimers/molecule results in 500 (±10%) transformants/ml of culture, while cultures transformed with plasmid molecules containing fewer dimers contain proportionately larger numbers of transformants according to the relationship S/S₀ = e⁻¹λ, where λ is the number of pyrimidine dimers/molecule.

For experiments to determine k₉₅ competing DNA was prepared by irradiating plasmid pBM150 (tet' amp') as described before (Johnston and Davis, 1984) at a concentration of 20 µg/ml with sufficient 254-nm light to produce 100 dimers/plasmid molecule. Plasmid DNA was concentrated by ethanol precipitation and the concentration was determined by absorbance at 260 nm.

Nitrocellulose Filter Binding Assay—The details of this assay have been published previously (Riggs et al., 1968; Madden et al., 1973; Seawell et al., 1980; G. Sancar et al., 1985). Briefly, the reaction mixture (110 µl) consisted of 50 mM Tris, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 100 µg/ml bovine serum albumin, DNA at the concentrations indicated in the figure legends, and 100 mM NaCl unless stated otherwise. After addition of photolyase to the desired concentration, the reaction mixture was incubated at 22 °C for 1 h before filtering through Schleicher and Schull B885 filters (24-mm diameter). All experiments were performed under illumination from G.E. gold fluorescent bulbs.

Flash Photolysis—This method is a modification of Porter's (1960) classic flash photolysis technique adapted by Harm and Rupert (1968) to the study of the action mechanism of photolyase. DNA-photolyase complexes present in a reaction mixture are subjected to a high intensity flash of photoreactivating light of approximately 1-ms duration, resulting in repair of pyrimidine dimers bound by the enzyme at the instant of the flash. Since the turnover rate of the enzyme is considerably longer than the duration of the flash, the number of dimers repaired (as determined by biological or chemical methods) is a measure of the number of ES complexes present at the moment of the flash. (See also Jorns et al., 1986, in this series.) Originally Rupert and colleagues used Yashika PRO 50 photographic flash units to provide the flash. We have found that any of several commercially available photographic flash units are equally applicable provided that the sample is screened with Pyrex glass and/or the plastic lid of the Petri dish to eliminate short wavelength radiation. In the studies described in this paper we used Vivitar 2500 photographic flash units for flash photoreactivation.

Substrate for the flash photolysis assay was UV-irradiated pBR322 DNA, labeled and irradiated as described above. Following flash photoreactivation, photolyase was removed by phenol extraction and the DNA was removed from photolyase-enzyme buffer and concentrated by ethanol precipitation. An aliquot of the DNA was then used in the transformation assay to determine the number of UV-induced lesions and pyrimidine dimers remaining per molecule. Losses during extraction and precipitation were monitored by scintillation counting.

RESULTS

Visualization of Photolyase-DNA Complexes

We have previously reported (G. Sancar et al., 1985) that photolyase binds specifically to UV-irradiated DNA and has little affinity for nonirradiated DNA. This is also apparent from analysis of DNA/photolyase mixtures by electron microscopy. Plasmid pBR322 DNA containing dimers only in a

FIG. 1. Binding of photolyase to linearized pBR322 DNA containing pyrimidine dimers in a slightly off-center location. The pBR322 substrate, prepared as described under “Materials and Methods,” was incubated with photolyase at a concentration of 1.8 nM DNA and 9.3 nM enzyme under standard binding conditions for 1 h, photoreactivating light was supplied as described above; a Sylvania F15T8SBL bulb for a fluence rate of 10 J/m². The DNA was then labeled with 32P to a concentration of 20 µg/ml with sufficient 254-nm light to produce 100 dimers/plasmid molecule. Plasmid DNA was concentrated by ethanol precipitation and the concentration was determined by absorbance at 260 nm.
lyase binding to DNA as a monomer (data not shown). This observation is in agreement with our previous results obtained from gel filtration studies which showed that photolyase elutes as a $M_r = 49,000$ protein (A. Sancar et al., 1984b). Because of difficulty in visualizing proteins as small as photolyase by electron microscopy we have not obtained a sufficient number of photographs to quantitate the specific and nonspecific association constants by this method; however, we may draw the qualitative conclusion from this and other micrographs that photolyase does not bind to nonirradiated DNA to any significant extent.

Quantitation of the Association Constant $K_A$ of Blue Photolyase

Nitrocellulose Filter Binding—The nitrocellulose filter binding technique first used to study Lac repressor-operator interactions has become a standard technique for quantitating enzyme-substrate complex formation for DNA-binding proteins (Riggs et al., 1968, 1970). We have utilized this technique to study the equilibrium binding of photolyase to UV-irradiated DNA. As can be seen in Fig. 2, incubation of radiolabeled pBR322 DNA containing an average of 5.5 pyrimidine dimers/molecule with increasing concentrations of photolyase results in increasing retention of the DNA by the nitrocellulose filters up to the point at which saturation is apparently obtained. However, the relationship between the fraction of total DNA retained on the filter and the total number of ES complexes present in solution is nonlinear in this case because 1) the efficiency of retention of molecules containing a single ES complex is less than 1.0 for photolyase-pyrimidine dimer complexes (G. Sancar et al., 1985) and 2) the substrate (pyrimidine dimers) is not distributed uniformly among the DNA molecules, but rather follows a Poisson distribution. As is described in the Appendix, beginning with the relationship derived by Woodbury and von Hippel (1983) for a substrate with multiple independent binding sites, we have derived the following equations which express the quantitative relationship between the fraction of total DNA retained by the filter ($R$), the retention efficiency ($\epsilon$), the average number of pyrimidine dimers/molecule ($\lambda$), the total protein concentration ($P_i$), the DNA concentration ($D$), and the association constant $K_A$:

$$R = 1 - e^{-\lambda \epsilon} \frac{K_A P}{1 + K_A P}$$

(1)

where

$$K_A P = K_A (P_i - ES) = \alpha + (\alpha^2 + K_A P_i)^{1/2}$$

(2)

and

$$\alpha = \frac{1}{2}(K_A P_i - (1 + \lambda K_A D)).$$

(3)

$K_A$ was determined from the filter binding data shown in Fig. 2 as follows: the known values for $P_i$, $D$, $\lambda$, and $\epsilon$ ($=0.34$; quantitated by titrating enzyme with dimer-containing DNA, G. Sancar et al., 1985) were used to generate theoretical curves of predicted retention ($R$) versus log $K_A$ for each protein concentration (Fig. 3). The experimentally observed retention at each protein concentration was then plotted on the appro-

1 The "Appendix" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 450 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1951, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
appropriate curve and the $K_A$ values thus obtained (Fig. 3) when averaged gave a value for $K_A = 6.0 \pm 2.1 \times 10^7$ M$^{-1}$ for the binding of photolyase to pyrimidine dimers in DNA. The good agreement between the observed retention of dimer-containing DNA and that predicted for this $K_A$ is shown by the fact that the solid curve drawn through the data points in Fig. 2 is the theoretical curve calculated from Equations 1–3 using $K_A = 6.0 \times 10^7$ M$^{-1}$, while the dashed lines indicate the retention expected for $K_A$ values of 1 standard deviation from the mean.

**Flash Photolysis**—As an increasing proportion of the substrate-binding sites on each DNA molecule are bound by photolyase, the nitrocellulose filter binding assay becomes less sensitive to increases in ES complexes/molecule; in addition, as can be seen in Fig. 3 and Equations 1–3, as saturation is approached small differences in the apparent fraction of DNA molecules retained make large differences in the apparent $K_A$. Therefore, we chose to use a second method to confirm our estimate of $K_A$, namely the flash photolysis technique originally used by Harm and Rupert (1968, 1970) to study binding of yeast photolyase in vitro. Various concentrations of photolyase were mixed with UV-irradiated DNA and after 1 h of incubation in the dark, the mixture was exposed to a single intense flash of photoreactivating light at the time of the flash. As can be seen in Fig. 4, a typical enzyme saturation plot is thus obtained. The analysis of the data by an Eadie-Scatchard plot (Segel, 1975) is shown in Fig. 4; photolyase binds to dimers with an apparent stoichiometry of 0.75 enzyme molecules/dimer and with an association constant $K_A = 4.7 \times 10^7$ M$^{-1}$ in good agreement with the value for $K_A$ obtained by the nitrocellulose filter binding technique. It should be noted that at great (200–2000-fold) enzyme excess, only about 75% of dimers are repaired by a single flash suggesting that some enzyme molecules are able to bind pyrimidine dimers but are deficient in photolysis (see below and G. Sancar et al., 1987, in this series).

**Binding of Yellow Photolyase to Pyrimidine Dimers**

*E. coli* photolyase contains a FAD neutral blue radical (Jorns et al., 1984) and has a blue-purple color when freshly prepared. Upon storage at $-20^\circ$C the radical is oxidized to FAD$_{ox}$ and the enzyme turns yellow. Since at present we are unable to oxidize the blue radical in an experimentally controlled manner, we do not have fully oxidized enzyme. However, we have obtained an enzyme preparation in which 70% of the blue radical has been converted to FAD$_{ox}$ (Jorns et al., 1987) after 6 months of storage at $-20^\circ$C. When we conducted the filter binding experiment with this enzyme preparation the results shown in Fig. 5 were obtained. The yellow enzyme preparation was still capable of binding specifically to UV-irradiated DNA, displaying an apparent association constant $K_A = 4.8 \times 10^7$ M$^{-1}$. Clearly the binding seen is in excess of that expected if only the remaining enzyme containing neutral blue radical were capable of binding DNA (Fig. 5, broken line).

**Dependence of $K_A$ on [NaCl]**

When proteins bind to DNA by ionic interactions between positively charged groups on the protein and negative charges on the DNA phosphate backbone, counter ions are displaced from the phosphates as the result of binding. Therefore, if an important fraction of the total binding energy is this ionic interaction, $K_A$ shows a strong dependence on the cation concentration in the reaction mixture. Record et al. (1976) have derived a quantitative relationship between $K_A$, ion pairs formed upon binding, and the counter ion concentration:

$$\frac{d\log K_A}{d\log [\text{counterion}]} = -m'\chi$$

(4)

where $\chi$ is the fraction of counterion [Na] bound per DNA.

![FIG. 4. Eadie-Scatchard plot of the equilibrium binding of photolyase to UV-irradiated pBR322 DNA as measured by the flash photolysis assay. $^{3}H$-pBR322 DNA at a concentration of 3.84 nm and containing an average of 5.1 pyrimidine dimers/molecule was incubated with photolyase at the indicated enzyme concentrations under standard reaction conditions. Formation of ES complexes was quantitated by the flash photolysis technique followed by the transformation assay as described. In this experiment 75% of pyrimidine dimers (14.7 nm) were repaired by a single flash at saturating enzyme concentrations; the concentration of ES complexes has been corrected in the inset and Figure to reflect this fact. The line is a linear least squares fit of the data obtained from the binding isotherm (also corrected for repair of 75% of ES complexes with a single flash) shown in the inset.](image1)

![FIG. 5. Binding of photolyase yellow enzyme to UV-irradiated pBR322 DNA. Incubation of photolyase with $^{3}H$-labeled UV-irradiated pBR322 DNA and quantitation of binding by the nitrocellulose filter binding assay were as described in the legend to Fig. 2 except that the DNA concentration was 1.7 nm and contained 6.0 pyrimidine dimers/molecule. The solid line shows binding expected for $K_A = 4.8 \pm 0.57 \times 10^7$ M$^{-1}$ obtained from this data, while the broken line indicates the binding expected if only the 30% of the enzyme containing the blue neutral radical is active in binding to dimers.](image2)
The binding of photolyase to DNA

| NaCl concentration (mM) | $K_a$ ($\pm$ kcal/mol) |
|-------------------------|------------------------|
| 125                     | 5.7 ± 1.7 $\times 10^7$|
| 200                     | 5.3 ± 2.1 $\times 10^7$|
| 275                     | 3.3 ± 1.8 $\times 10^7$|
| 350                     | 3.8 ± 1.7 $\times 10^7$|
| 400                     | 9.5 ± 4.4 $\times 10^6$|
| 450                     | 7.1 ± 2.5 $\times 10^6$|
| 500                     | 1.2 ± 0.24 $\times 10^6$|

To determine the dissociation rate constant $k_2$, we utilized the "cold DNA chase" method of Riggs et al. (1970) and Harm and Rupert (1970). Photolyase was incubated with UV-irradiated pBR322 DNA and incubated for 1 h to allow binding to reach equilibrium; competing pBM150 DNA containing a 20-fold molar excess of pyrimidine dimers compared to the pBR322 DNA was then added and at various later times samples were removed and exposed to flash photolysis thus repairing dimers in ES complexes at the time of the flash. Quantitation of ES complexes was by the transformation assay selecting for tetracycline-resistant transformants. Ideally under the reaction conditions described above, the term $k_2[E][S]$ in Equation 6 becomes negligible and thus $k_2$ can be determined from the equation

$$\ln \frac{[ES]}{[ES]_0} = -k_2 t$$

where $[ES]_0$ is the concentration of complexes immediately prior to the addition of competitor and $[ES]$, is the concentration of complexes at some later time $t$ after addition of competitor. If competition is 100% efficient and if all complexes have the same $k_2$ then a semilog plot of $\ln [ES]/[ES]_0$ versus $t$ should yield a straight line with a slope of $-k_2$ until the system nears the new equilibrium state at which a small number of ES complexes remain bound to dimers on pBR322.

To correct for this latter effect we have subtracted the fraction of ES complexes remaining at 60 and 120 min (28%) from [ES] and [ES]$_0$ on the assumption that this approximates the

**TABLE I**

| Effect of [NaCl] on $K_a$ of E. coli photolyase |
|-----------------------------------------------|
| NaCl concentration (mM) | $K_a$ ($\pm$ kcal/mol) |
|-------------------------|------------------------|
| 125                     | 5.7 ± 1.7 $\times 10^7$|
| 200                     | 5.3 ± 2.1 $\times 10^7$|
| 275                     | 3.3 ± 1.8 $\times 10^7$|
| 350                     | 3.8 ± 1.7 $\times 10^7$|
| 400                     | 9.5 ± 4.4 $\times 10^6$|
| 450                     | 7.1 ± 2.5 $\times 10^6$|
| 500                     | 1.2 ± 0.24 $\times 10^6$|

**Fig. 6.** Dependence of the association constant $K_a$ on NaCl concentration. The binding constant at each NaCl concentration was determined as described in the legends to Figs. 2 and 3 except that the reaction mixture contained 10 mM Tris, pH 7.5, and NaCl at the appropriate concentrations. The error bars indicate the standard deviation of the mean for each $K_a$ determination. The line was obtained by linear least squares regression analysis which indicated a correlation coefficient of 0.87. $m'$ is the slope and equals the number of protein-phosphesteri backbone when bound to the dimer.

**Fig. 7.** Determination of $k_2$ by the decrease of ES complexes in pBR322 in the presence of competing substrate. UV-irradiated pBR322 DNA containing an average of 4.2 dimers/molecule was incubated at a concentration of 3.3 mM with photolyase at 70 nM under standard reaction conditions in the dark for 1 h. Competing UV-irradiated DNA (pBM150 containing approximately 100 dimers/ genome) was added at a concentration of 2.66 mM at zero time; at various later times samples were taken and the number of ES complexes remaining were determined by flash photolysis and transformation assay. $k_2$ was determined by extrapolation of the initial slope (15- and 30-s time points) to a value of 0.37.
new equilibrium value (data not shown). The results of a typical experiment are shown in Fig. 7 from which it can be seen that 1) a large fraction of the complexes dissociate very quickly and 2) the decrease in the fraction of [ES] complexes with time is not linear. We interpret this latter result as reflecting the presence of at least 2 classes of [ES] complexes with respect to dissociation rate. The rapidly dissociating class comprises approximately 65% of ES complexes present at time 0 and (from this and three other experiments) has an estimated $k_2 = 2-3 \times 10^{-2} \text{ s}^{-1}$ while the remaining complexes dissociate with an apparent rate constant $k_1 = 1.3 \times 10^{-3}$ to $6 \times 10^{-4} \text{ s}^{-1}$. The presence of two classes of ES complexes which differ in their kinetic parameters is not unexpected as Harm (1970a, 1970b) obtained similar results for the dissociation of ES complexes present at each time point. Using values for $k_1$ of $1.4 \times 10^{-6}$ to $4.2 \times 10^{-6} \text{ M}^{-1} \text{s}^{-1}$ and for $k_2$ of $3 \times 10^{-2} \text{ s}^{-1}$ we calculate $K_A = 4.7 \times 10^{7} \text{ M}^{-1}$ to $1.4 \times 10^{6} \text{ M}^{-1}$ in good agreement with the values of $4.7 \times 10^{8}$ to $6 \times 10^{7}$ obtained by equilibrium binding.

**DISCUSSION**

Photolyase is a spherical molecule of about 50 Å diameter which binds specifically to irradiated DNA. Since the enzyme does not act on photoproducts other than pyrimidine dimers (Brash et al., 1985) we conclude that this specificity is the result of photolyase-pyrimidine dimer binding. Perhaps the most interesting and surprising result of this study is the relatively minor contribution of electrostatic interactions to the binding of photolyase to substrate. The dependence of $K_A$ on monovalent cation concentration indicates that the protein makes electrostatic contacts with only 1–2 phosphates on the DNA backbone. This conclusion is consistent with other observations: (i) the enzyme can act efficiently on substrates as small as oligo(dT)$_6$ (Jorns et al., 1985); (ii) photolyase does not inhibit the incision by ABC excision nuclelease of the fourth phosphodiester bond 3' to a pyrimidine dimer (A. Sancar et al., 1984a). The fact that only about 10% of the total free energy of photolyase-DNA binding is electrostatic at moderate salt concentrations has important implications for the specificity of binding and for the mechanism by which the enzyme "searches" for its target.

At optimum conditions of ionic strength and pH, all formerly characterized sequence-specific DNA binding proteins (e.g. lac repressor and EcoRI restriction endonuclease) have $K_A$ values $\geq 10^{11} \text{ M}^{-1}$ (see Berg and von Hippel, 1985; Terry et al., 1983), consistent with the selectivity of these proteins; in contrast we find $K_A = 6 \times 10^{6} \text{ M}^{-1}$ for E. coli photolyase, yet in vivo data indicate that the enzyme is highly specific. Harm (1970b) reported that in E. coli cells containing approximately 20 pyrimidine dimers and 20 photolyase molecules, about 10 enzyme-substrate complexes were present at equilibrium. If we assume that all of the remaining enzyme molecules bound nonspecifically and that the photolyase binding site spans 4 nucleotides (Jorns et al., 1985), then the minimum specificity of photolyase binding (defined by the ratio of the specific to nonspecific association constants) is $8 \times 10^{4}/4 \times 10 = 2 \times 10^{3}$ (assuming that each of the 8 $\times 10^{5}$ bases in the E. coli chromosome constitutes the beginning of a potential nonspecific binding site). This value is similar to those reported for sequence-specific binding proteins; thus it is the ratio of specific to nonspecific binding constants rather than the absolute values of these constants that is important in determining specificity (von Hippel and Berg, 1986). The major difference between E. coli photolyase and the lac re-
pressor or EcoRI is that the latter two proteins have large nonspecific association constants mediated largely or entirely by electrostatic interactions with DNA phosphates, and therefore must also have high $K_a$ values to retain specificity (Winter and von Hippel, 1981; de Haseth et al., 1977; Revzin and von Hippel, 1977; Berg and von Hippel, 1985; Terry et al., 1983; Jen-Jacobson et al., 1983). The S187 mutant of the EcoRII protein makes 2 phosphate contacts at pH 7.4 compared to 8 phosphate contacts for the wild type enzyme; the result is that while $K_a$ for the enzyme decreases from $10^{11}$ M$^{-1}$ (wild type) to $4.6 \times 10^8$ M$^{-1}$ (S187), the enzyme retains its specificity for binding to the EcoRI recognition sequence (Jen-Jacobson et al., 1983), indicating that the specificity of EcoRII binding, like photolyase, derives largely from nonionic interactions.

A number of sequence-specific DNA-binding proteins utilize nonspecific electrostatic interactions to increase the kinetic efficiency of binding; the high association rate constants of proteins such as lac repressor generally exceed the Smoluchowski limit for diffusion controlled reactions (Riggs et al., 1970; Berg et al., 1981; Berg and von Hippel, 1985) indicating that the proteins find their targets by one-dimensional or facilitated diffusion. Our results indicate that the contribution of such mechanisms of reduced dimensionality are much less important for binding of photolyase than for other DNA-binding proteins, as the bimolecular association constant $k_1 = 4.5 \times 10^9$ M$^{-1}$ s$^{-1}$ is well within the limit of a diffusion controlled reaction for a spherical protein of 50 Å diameter. Furthermore, the minor contribution of electrostatic interactions to photolyase binding argues against the use of unidimensional diffusion as a protein must have a significant level of nonspecific, electrostatic binding to slide or track along DNA (Berg and von Hippel, 1985). The above arguments do not rule out relatively small contributions of nonspecific interactions to both the kinetics of photolyase binding and the intracellular equilibrium; indeed it is clear that some nonspecific binding of the enzyme to DNA does occur as minicells of E. coli which lack DNA are devoid of photolyase (Setlow and Cohen cited in Cohen et al., 1968) while minicells containing R factor DNA have a small but detectable amount of photoreactivating enzyme (Paterson and Roozen, 1972). However, the magnitude of these effects relative to lac repressor or EcoRI must be small.

It is of interest to compare the values which we obtain in vitro for the kinetic and equilibrium constants for photolyase binding with values obtained in vivo by Harm (1970a). Because the photolysis reaction can easily be separated from the binding reaction in vivo as well as in vitro this enzyme offers a unique opportunity to measure reaction parameters under both conditions and to compare them. Using the flash photolysis technique in vivo, Harm (1970a) found $k_1 = 1.1 \times 10^6$ M$^{-1}$ s$^{-1}$, $k_2 = 1.3 \times 10^{-2}$ to $1.9 \times 10^{-3}$ s$^{-1}$ from which $K_a$ can be calculated to be $8.5 \times 10^5$ to $5.6 \times 10^6$ M$^{-1}$. All of these values agree reasonably with the in vitro values reported here: $k_1 = 1.4-4.2 \times 10^6$ M$^{-1}$ s$^{-1}$, $k_2 = 2-3 \times 10^{-2}$ s$^{-1}$, and $K_a = 6.0 \times 10^3$ M$^{-1}$. Thus we conclude that the kinetic and equilibrium constants we obtain for photolyase binding in vitro reflect the in vivo state.

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The starting point of our derivation is the following relationship between \( A_{\text{free}} \) and \( A_{\text{bound}} \) (eq. 48):

\[
B = 1 - \left( 1 + N^a \right)^{-1}
\]

where \( N \) is the fraction of total DNA molecules, \( a \) is the intrinsic binding constant that is associated with each binding site \((E)\), \( B \) is the free protein concentration, \( a \) is the probability that a single protein is bound at a particular site and \( k \) is defined as the equilibrium constant for the association of \( a \) sites to the entire DNA molecule. The number of binding sites \( n \) per DNA molecule is calculated as \( N \) times the average number of protein binding sites per DNA molecule. Equation 3 is derived for a population of DNA and protein molecules with the following assumptions: 1) each protein molecule contains a single DNA binding site; 2) each protein molecule contains the same number of potential binding sites, for each protein molecule contains the same number of potential DNA binding sites, and each DNA molecule contains the same number of potential protein binding sites, 3) the equilibrium constant for the association of \( a \) sites to the entire DNA molecule is defined as \( K \), and 4) the equilibrium constant for the association of \( a \) sites to the entire DNA molecule is defined as \( K \). For our system, that for every DNA molecule contains exactly \( a \) binding sites, this fact further implies that at saturating protein concentrations bound protein molecules will be present in a distribution which is different from that dependent upon the association distribution of protein dimers. The problem then is to derive a relationship between \( B \) and \( K \), where \( B \) is the equilibrium association constant which takes the different distributions into account.

It follows from equation (A3) above and from the assumption of equal and independent binding sites that for each class of protein molecules containing exactly \( a \) binding sites, (per molecule):

\[
B = 1 - \left( 1 + N^a \right)^{-1}
\]

and then for the total population of protein molecules:

\[
B = 1 - \left( 1 + N^a \right)^{-1}
\]

where \( N \) is the fraction of DNA molecules having exactly \( a \) binding sites. Since the ratio of DNA molecules with exactly \( a \) binding sites according to a Poisson distribution:

\[
N = e^{-\lambda}
\]

and

\[
N^a = e^{-\lambda a}
\]

where \( \lambda \) is the mean number of binding sites per DNA molecule for the entire population of protein molecules. Thus the observed \( B \) for a substrate with binding sites \( a \) is a Poisson distribution:

\[
B = e^{-\lambda}
\]

and

\[
B = e^{-\lambda a}
\]

where 1 - \( e^{-\lambda a} \) equals the linear binding efficiency \( a \) = 1 - \( e^{-\lambda a} \)

\[
B = e^{-\lambda a}
\]