

Action Mechanism of *Escherichia coli* DNA Photolyase

I. FORMATION OF THE ENZYME-SUBSTRATE COMPLEX*

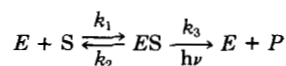
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Escherichia coli DNA photolyase (photoreactivating enzyme) is a flavoprotein. The enzyme binds to DNA containing pyrimidine dimers in a light-independent step and, upon illumination with 300–600 nm radiation, catalyzes the photosensitized cleavage 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 1.4×10^6 to $4.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The dissociation of the enzyme from dimer-containing DNA displays biphasic kinetics; for the rapidly dissociating class of complexes $k_2 = 2-3 \times 10^{-2} \text{ s}^{-1}$, while for the more slowly dissociating class $k_2 = 1.3 \times 10^{-3}$ to $6 \times 10^{-4} \text{ s}^{-1}$. The equilibrium association constant K_A , as determined by the nitrocellulose filter binding assay and the flash photolysis assay, was 4.7×10^7 to $6 \times 10^7 \text{ M}^{-1}$, 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 unrepaired 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.



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

Escherichia coli (Rupert *et al.*, 1958) and the action of the enzyme has been studied extensively *in vivo* (Harm *et al.*, 1968; Harm, 1970a, 1970b), until recently the paucity of the enzyme (about 20 molecules/cell in wild type cells) had made *in vitro* studies of the enzyme in a fully defined system impossible. Several years ago we began a concentrated effort to obtain sufficient protein to undertake studies on the molecular mechanism of action of photolyase; to that end we cloned the *phr* gene (Sancar and Rupert, 1978b), constructed overproducing *E. coli* strains in which >15% of total cellular protein is photolyase, and purified the enzyme to homogeneity (A. Sancar *et al.*, 1984b). Pure photolyase consists of an M_r -53,994 polypeptide (G. Sancar *et al.*, 1984) and two intrinsic chromophores, a blue neutral FAD radical and a second chromophore the chemical identity of which is not yet known (Sancar and Sancar, 1984; Jorns *et al.*, 1984); *in vitro* the enzyme is capable of repairing pyrimidine dimers in the absence of any additional proteins or cofactors and at a rate similar to that observed *in vivo*. Using this fully defined system we report in this and the following two papers the results of studies on the thermodynamic, kinetic, and photochemical processes involved in photoreactivation by the *E. coli* enzyme. In this article we deal with the first half of the reaction sequence, formation of the enzyme-substrate complex.

We have previously reported that purified photolyase binds specifically to UV-irradiated DNA and that this binding is relatively insensitive to changes in salt concentration or pH (G. Sancar *et al.*, 1985). In this report we extend those studies by quantitating the specific association constant K_A using both the nitrocellulose filter binding assay and the flash photolysis-transformation technique; in addition we have quantitated the kinetic constants for complex formation, k_1 and k_2 . Both equilibrium and kinetic methods yield estimates for K_A of 4.7×10^7 to $1.4 \times 10^8 \text{ M}^{-1}$, values which are unusually low for a site-specific DNA-binding protein. In addition, quantitation of the effect of NaCl concentration on K_A indicates that the enzyme contacts only 1–2 phosphates on the DNA backbone when bound to the pyrimidine dimer; taken together with the relatively low k_1 ($4.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) exhibited by the enzyme, these results suggest that in contrast to other site-specific DNA-binding proteins, nonspecific ionic interactions contribute very little to the equilibrium or kinetics of photolyase binding.

MATERIALS AND METHODS

Enzyme Preparations—*E. coli* DNA photolyase was purified to homogeneity as described previously (A. Sancar *et al.*, 1984b) with the modification that at Step VI, Blue Sepharose (Sigma) was substituted for DNA cellulose; this change enabled us to obtain pure enzyme at higher yield (about 10% versus 5.4%) than previously. The enzyme had a blue-purple color when freshly prepared due to its neutral FAD radical cofactor (Jorns *et al.*, 1984) and concentrated

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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 analysis (absorption at 450 and 580 nm) indicated 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*)₃₇₅ and (*BamHI*-*EcoRI*)₃₉₈₈ fragments were separated on agarose gels and purified as described by Vogelstein and Gillespie (1979). The small fragment was irradiated with 1.5 kJ/m^2 of 254 nm of light to produce an average of 3 pyrimidine dimers/molecule. The irradiated DNA was mixed with the nonirradiated (*BamHI*-*EcoRI*)₃₉₈₈ fragment at a concentration of $4\text{ }\mu\text{g/ml}$ 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 (4363 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.*, 1985). The specific activity was 1.3×10^5 cpm/ μg . The DNA was diluted to a concentration of $20\text{ }\mu\text{g/ml}$ 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.*, 1985); incubation of UV-irradiated DNA with a molar excess of photolyase for 1 h under photoreactivating light (supplied by a Sylvania F15TF8BLB bulb at a fluence rate of approximately 10 J/m^2), followed by transformation was used to quantitate the average number of pyrimidine dimers/molecule. When performed as described, the transformation assay is a sensitive and accurate method for quantitating dimer repair. Typically transformation with $0.1\text{ }\mu\text{g}$ of plasmid DNA containing an average of 6 pyrimidine dimers/molecule results in $500 (\pm 10\%)$ transformants/ml of culture, while cultures transformed with plasmid molecules containing fewer dimers contain proportionately larger numbers of transformants according to the relationship $S/S_0 = e^{-\lambda}$, where λ is the number of pyrimidine dimers/molecule.

For experiments to determine k_2 , competing DNA was prepared by irradiating plasmid pBM150 (*tet*^r *amp*^r; Johnston and Davis, 1984) at a concentration of $20\text{ }\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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 BA85 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 a 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 assay 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

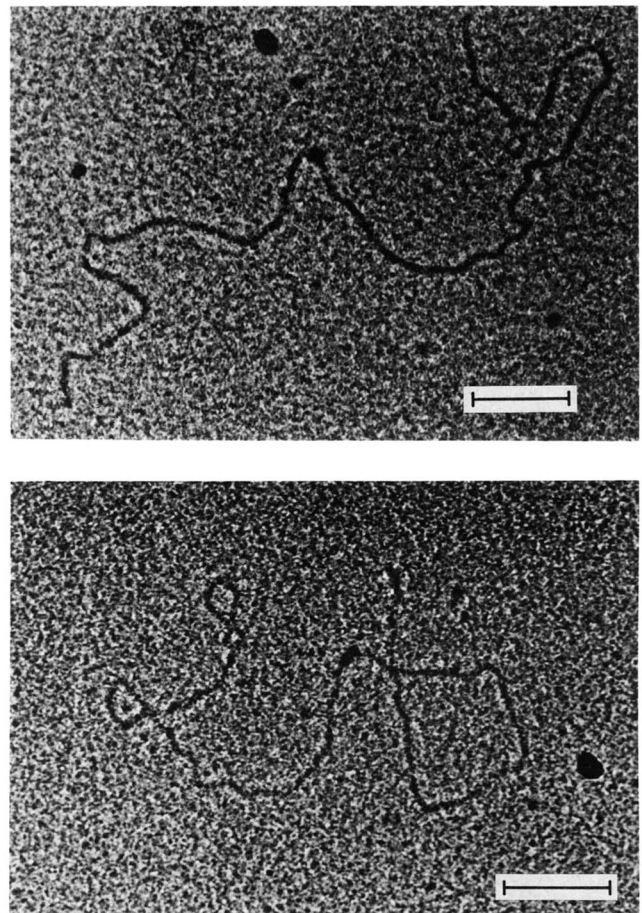


FIG. 1. Binding of photolyase to linearized pBR322 DNA containing pyrimidine dimers in a slightly off-center location. The pBR322 substrate, prepared as described under “Materials and Methods,” was incubated with photolyase at a concentration of 1.8 nM DNA and 9.3 nM enzyme under standard binding conditions for 1 h; photolyase-DNA complexes were then fixed and processed for transmission electron microscopy as previously described (Griffith and Christiansen, 1978). Bar indicates 100 nm. Upper panel shows one photolyase-DNA complex while the DNA molecule in the lower panel appears to bear two complexes; it should be noted that less than 10% of the molecules analyzed contained two ES complexes.

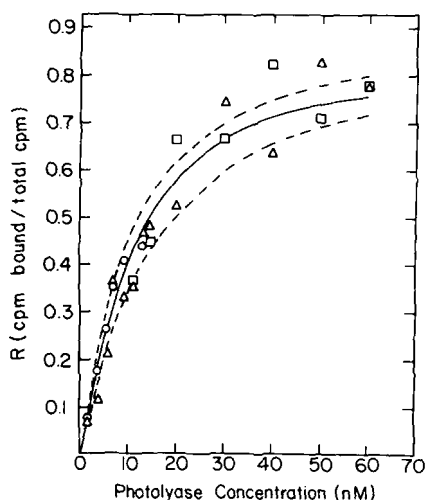


FIG. 2. Binding of photolyase (blue enzyme) to ^3H -labeled UV-irradiated pBR322 DNA. Photolyase at the indicated concentrations was incubated with ^3H -labeled pBR322 DNA containing an average of 5.5 pyrimidine dimers/molecule and at a plasmid concentration of 2.34 nM. Following 1-h incubation in the dark, duplicate samples were filtered through nitrocellulose and the counts retained were determined by scintillation counting. Symbols: \circ , Δ , and \square , values obtained in three independent experiments. The solid line drawn through the data points is the expected retention for $K_A = 6.0 \times 10^7 \text{ M}^{-1}$, while the dashed lines show the expected retention for $K_A = 8.1 \times 10^7 \text{ M}^{-1}$ (top) and $3.9 \times 10^7 \text{ M}^{-1}$ (bottom).

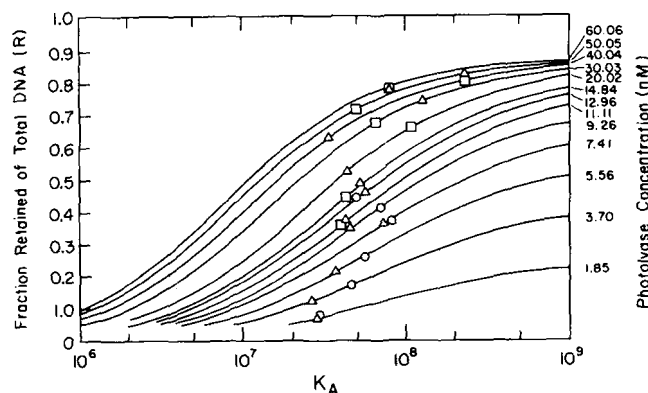


FIG. 3. Expected retention (R) by nitrocellulose filters of UV-irradiated pBR322 DNA as a function of the association constant (K_A) for photolyase-pyrimidine dimer binding. Equations 1-3 were used to generate curves showing expected retention at each of the indicated photolyase concentrations for binding of pBR322 DNA at a concentration of 2.34 nM and containing 5.5 pyrimidine dimers/molecule; a value of 0.34 was used for ϵ in Equation 1 (G. Sancar *et al.*, 1985). The data points shown in Fig. 2 were then plotted on these curves to obtain the K_A values. Symbols are the same as described in the legend to Fig. 2.

374-base pair region in a slightly off-center location relative to the ends of the linearized molecule was mixed with photolyase in the dark and examined by electron microscopy. Fig. 1 shows two representatives of the majority of ES complexes we observed. The enzyme binds with measurable frequency only to the irradiated region and binding to this region is not seen if the mixture is exposed to photoreactivating light prior to fixing the complexes with glutaraldehyde (not shown). From the micrographs shown in Fig. 1 it is apparent that photolyase is a spherical molecule of about 50 Å diameter; furthermore, under the same conditions and magnification, DNA polymerase I ($M_r = 103,000$) and UvrA protein ($M_r = 104,000$) appear to be about twice the size of photolyase ($M_r = 53,994$; G. Sancar *et al.*, 1984) consistent with photo-

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,¹ 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 (ϵ), the average number of pyrimidine dimers/molecule (λ), the total protein concentration (P_t), the DNA concentration (D), and the association constant K_A :

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

where

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

and

$$\alpha = 1/2 [K_A P_t - (1 + \lambda K_A D)]. \quad (3)$$

K_A was determined from the filter binding data shown in Fig. 2 as follows: the known values for P_t , D , λ , and ϵ ($=0.34$; 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, 9650 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 $K_A = 6.0 \pm 2.1 \times 10^7 \text{ M}^{-1}$ 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 $K_A = 6.0 \times 10^7 \text{ M}^{-1}$, 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 sufficient to repair all dimers complexed with active enzyme; determination by transformation assay of the number of dimers/DNA molecule before and after the flash provides a direct measure of the number of ES complexes present at the time of the flash. As can be seen in Fig. 4, a typical enzyme saturation plot is thus obtained. 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

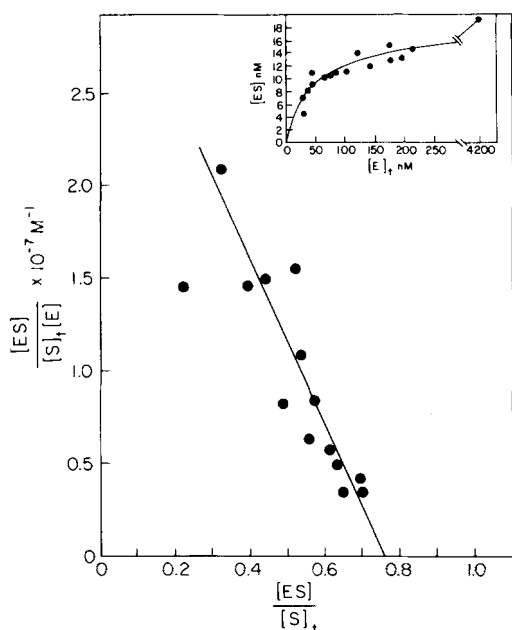


FIG. 4. Eadie-Scatchard plot of the equilibrium binding of photolyase to UV-irradiated pBR322 DNA as measured by the flash photolysis assay. ^3H -pBR322 DNA at a concentration of 3.84 nM and containing an average of 5.1 pyrimidine dimers/molecule was incubated with photolyase at the indicated enzyme 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*.

enzyme molecules/dimer and with an association constant $K_A = 4.7 \times 10^7 \text{ M}^{-1}$ 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°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 neutral radical has been converted to FAD_{ox} (Jorns *et al.*, 1987) after 6 months of storage at -20°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 $K_A = 4.8 \times 10^7 \text{ M}^{-1}$. 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 $[\text{NaCl}]$

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:

$$\frac{d \log K_A}{d \log [\text{M}^+]} = -m' \chi \quad (4)$$

where χ is the fraction of counterion $[\text{Na}]$ bound per DNA

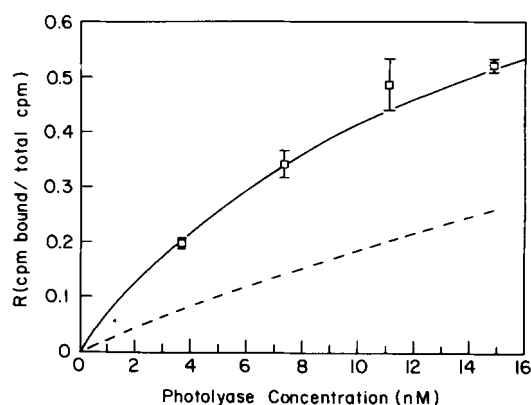


FIG. 5. Binding of photolyase yellow enzyme to UV-irradiated pBR322 DNA. Incubation of photolyase with ^3H -labeled UV-irradiated pBR322 DNA and quantitation of binding by the nitrocellulose filter binding assay were as described in the legend to Fig. 2 except that the DNA concentration was 1.7 nM and contained 6.0 pyrimidine dimers/molecule. The *solid line* shows binding expected for $K_A = 4.8 \pm 0.57 \times 10^7 \text{ M}^{-1}$ 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.

TABLE I
Effect of [NaCl] on K_A of *E. coli* photolyase

NaCl concentration <i>mM</i>	K_A
125	$5.7 \pm 1.7 \times 10^7$
200	$5.3 \pm 2.1 \times 10^7$
275	$3.3 \pm 1.8 \times 10^7$
350	$3.8 \pm 1.7 \times 10^7$
400	$9.5 \pm 4.4 \times 10^6$
450	$7.1 \pm 2.5 \times 10^6$
500	$1.2 \pm 0.24 \times 10^7$

phosphate (=0.88, Lohman *et al.*, 1980) and m' is the number of ion pairs formed between the DNA and protein. Table I shows K_A for photolyase binding as a function of [NaCl] as determined by the filter binding assay. From the slope of the Record-Lohman plot (Fig. 6) we obtain $m' = 1.6 \pm 0.3$. These results indicate that photolyase makes ionic contact with only 1–2 phosphates in the phosphodiester backbone when bound to the dimer.

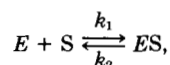
Extrapolation of K_{obs} to 1M NaCl concentration can be used to estimate the nonelectrostatic contribution (ΔG_{ne}) to the total free energy of photolyase-DNA binding (Record *et al.*, 1976). From the data shown in Table I and Fig. 6 we obtain $\Delta G_{1M} = -8.9$ kcal/mol at 20 °C. Since:

$$\Delta G_{ne} = \Delta G_{1M} - m' \Delta G_{lys} \quad (5)$$

(where $\Delta G_{lys} = 0.2$ kcal/mol, the standard free energy of a lysine-phosphate ionic interaction at 1 M NaCl), we obtain $\Delta G_{ne} = -9.2$ kcal/mol. Under our standard binding conditions $\Delta G = -10.5$ kcal/mol and thus only about 10% of the total free energy of binding of photolyase to a dimer-containing region is due to ionic interactions.

Determination of the Rate Constants k_1 and k_2

From the reaction scheme for photolyase-substrate binding



the change in concentration of ES complexes in the absence of photolysis is given by the differential equation

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] \quad (6)$$

where $[E]$ and $[S]$ are the concentrations of free enzyme and free substrate. Because of the complexities involved in trying to directly quantitate $[ES]$ by nitrocellulose filter binding in our system (see "Determination of K_A by Nitrocellulose Filter Binding," above) we have utilized the flash photolysis and transformation techniques to quantitate photolyase-pyrimidine dimer binding.

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 $k_1[E][S]$ in Equation 6 becomes negligible and thus k_2 can be

determined from the equation

$$\ln \frac{[ES]_t}{[ES]_0} = -k_2 t \quad (7)$$

where $[ES]_0$ is the concentration of complexes immediately prior to the addition of competitor and $[ES]_t$ 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 $\ln [ES]_t/[ES]_0$ 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 $[ES]_0$ and $[ES]_t$ on the assumption that this approximates the

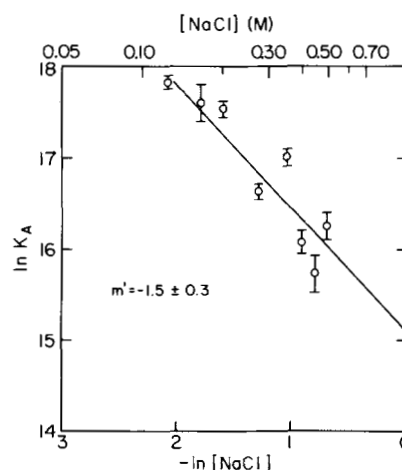


FIG. 6. Dependence of the association constant K_A on NaCl concentration. The binding constant at each NaCl concentration was determined as described in the legends to Figs. 2 and 3 except that the reaction mixture contained 10 mM Tris, pH 7.5, and NaCl at the appropriate concentrations. The error bars 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-phosphodiester bond contacts, as described in the text.

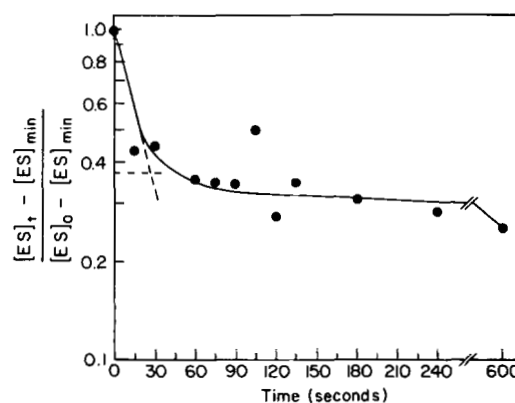


FIG. 7. Determination of k_2 by the decrease of ES complexes in pBR322 in the presence of competing substrate. UV-irradiated pBR322 DNA containing an average of 4.2 dimers/molecule was incubated at a concentration of 3.3 nM with photolyase at 70 nM under standard reaction conditions in the dark for 1 h. Competing UV-irradiated DNA (pBM150 containing approximately 100 dimers/genome) was added at a concentration of 2.66 nM 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.

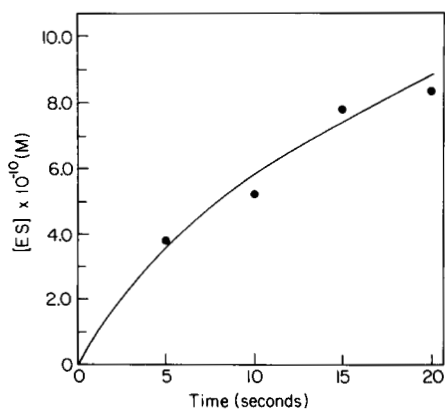


FIG. 8. Kinetics of formation of photolyase-pyrimidine dimer complexes. Reaction mixtures consisted of 0.45 nM pBR322 DNA containing an average of 5.4 pyrimidine dimers/molecule (= 2.43 nM pyrimidine dimers) in standard reaction buffer. Reactions were begun ($t = 0$) by addition of photolyase to 8.2 nM; at the indicated times the reaction mixture was subjected to flash photolysis and the number dimers repaired (and hence the number of ES complexes present at the time of the flash) were quantitated by the transformation assay.

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 $k_2 = 1.3 \times 10^{-3}$ 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 step *in vivo*. In addition, we have noted a marked variation in the rate of repair of specific pyrimidine dimers which appears to be dependent upon both dimer type and the surrounding sequences.²

A strategy similar to that for determining k_2 is frequently used to quantitate k_1 , i.e. reaction conditions are chosen such that the term $k_2[ES]$ in Equation 6 is negligible; thus quantitation of $[ES]$ as a function of time after mixing the reactants leads to an estimate of k_1 using the integrated rate equation for a simple bimolecular reaction.

$$\frac{1}{[E]_0 - [S]_0} \ln \frac{[S]_0 ([E]_0 - [ES]_t)}{[E]_0 ([S]_0 - [ES]_t)} = k_1 t \quad (8)$$

Alternatively, if conditions cannot be found such that $k_2[ES]$ is negligible then k_1 can be determined from the integrated form of Equation 6:

$$\ln \frac{2k_1[ES]_t + b - \sqrt{-q}}{2k_1[ES]_t + b + \sqrt{-q}} - \ln \frac{b - \sqrt{-q}}{b + \sqrt{-q}} = t \quad (9)$$

where

$$-b = k_1[S]_0 + k_1[E]_0 + k_2 \quad (10)$$

and

$$q = 4k_2^2[E]_0[S]_0 - b^2 \quad (11)$$

Fig. 8 shows the kinetics of ES complex formation determined

² G. Myles, B. van Houten, and A. Sancar, unpublished observations.

by adding photolyase to a reaction mixture containing UV-irradiated pBR322 DNA and subjecting the mixture to flash photolysis at various later times. As can be seen, even using the minimum concentrations of enzyme and substrate which allow us to reliably quantitate $[ES]$ by transformation, the reaction is very rapid. Only for the 5-s point shown in Fig. 8 is $k_1[E]_t[S]_t$ less than 20% of $k_2[ES]_t$ (using $k_2 = 3 \times 10^{-2} \text{ s}^{-1}$); using Equation 8 and this first point we obtain a value for $k_1 = 4.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. If, on the other hand, we use Equation 9 to determine k_1 , the values obtained are dependent upon the value chosen for k_2 as well as the proportion of the fast and slowly dissociating complexes present at each time point. Utilizing Equations 9-11 and $k_2 = 3 \times 10^{-2}$ we obtain a value for $k_1 = 1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

It is of interest to compare the equilibrium association constant K_A predicted from the relationship $K_A = k_1/k_2$ with the value obtained by the equilibrium binding experiments described above. Using values for k_1 of 1.4×10^6 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 $1.4 \times 10^8 \text{ M}^{-1}$, in good agreement with the values of 4.7×10^7 to 6×10^7 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)₃ (Jorns *et al.*, 1985); (ii) photolyase does not inhibit the incision by ABC excision nuclease 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 $K_A = 6 \times 10^7 \text{ M}^{-1}$ 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 $8 \times 10^6 / 4 \times 10 = 2 \times 10^5$ (assuming that each of the 8×10^6 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-

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- 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 *EcoRI* 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 $4.6 \times 10^8 \text{ M}^{-1}$ (S187), the enzyme retains its specificity for binding to the *EcoRI* recognition sequence (Jen-Jacobson *et al.*, 1983), indicating that the specificity of *EcoRI* 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 exceeds the Smolouchowski 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 $k_1 = 4.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ 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 $k_1 = 1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_2 = 1.3 \times 10^{-2}$ to $1.9 \times 10^{-3} \text{ s}^{-1}$ from which K_A can be calculated to be 8.5×10^7 to $5.6 \times 10^8 \text{ M}^{-1}$. All of these values agree reasonably with the *in vitro* values reported here: $k_1 = 1.4\text{--}4.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_2 = 2\text{--}3 \times 10^{-2} \text{ s}^{-1}$, and $K_A = 6.0 \times 10^7 \text{ M}^{-1}$. 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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Appendix to
Action Mechanism of *E. coli* DNA Photolyase I.
Formation of the Enzyme-Substrate Complex
by
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The starting point of our derivation is the following relationship derived by Woodbury and von Hippel (1963) to describe the observed retention of DNA-protein complexes by nitrocellulose filters:

$$R = 1 - \frac{(1 + kP\zeta)^n}{(1 + kP)^n} \quad (\text{eq. A1})$$

where R is the fraction of total DNA molecules retained, k is the intrinsic binding constant associated with each binding site ($=K_A$), P is the free protein concentration, ζ is the probability that a single protein complexed at a particular site is not retained by the filter and is equal to $1-\epsilon$ where ϵ is the observed filter binding efficiency, and n is the number of protein binding sites per DNA molecule. Equation 1 is derived for a population of DNA and protein molecules with the following assumptions: (a) each protein molecule contains a single DNA binding site; (b) each potential protein binding site on each DNA molecule is "equal and independent"; and (c) each DNA molecule contains the same number of potential protein binding sites. We have shown in this paper that for *E. coli* DNA photolyase assumption (a) is valid; furthermore we assume that (b) is valid realizing that in fact binding of photolyase is probably influenced to some degree by the surrounding nucleotide sequence (this paper). Assumption (c) is clearly not valid for our system as the substrate consists of a population of DNA molecules containing pyrimidine dimers in a Poisson distribution; this fact further implies that at saturating protein concentrations bound protein molecules will be present in a distribution which is different from but dependent upon the Poisson distribution of pyrimidine dimers. The problem then is to derive a relationship between R and K_A (equilibrium association constant) which takes the Poisson distributions into account.

It follows from equation (A1) above and from the assumption of equal and independent binding sites that for each class of plasmid molecules containing exactly n binding sites (pyrimidine dimers)

$$R_n = 1 - \frac{(1 + K_A P \zeta)^n}{(1 + K_A P)^n} \quad (\text{eq. A2})$$

and thus for the total population of plasmid molecules

$$R = \sum_{n=0}^{\infty} g_n R_n = \sum_{n=0}^{\infty} g_n \left[1 - \frac{(1 + K_A P \zeta)^n}{(1 + K_A P)^n} \right] \quad (\text{eq. A3})$$

where g_n is the fraction of DNA molecules having exactly n binding sites. Since the binding sites are distributed according to a Poisson distribution,

$$g_n = \frac{\lambda^n e^{-\lambda}}{n!} \quad (\text{eq. A4a})$$

$$\sum_{n=0}^{\infty} g_n = 1 \quad (\text{eq. A4b})$$

where λ is the mean number of binding sites per DNA molecule for the entire population of plasmid molecules. Thus the observed R for a substrate with binding sites in a Poisson distribution is

$$R = 1 - e^{-\lambda \zeta} \quad (\text{eq. A5})$$

$$\text{where } \tau = 1 - \frac{1 + K_A P \zeta}{1 + K_A P} \quad (\text{eq. A6})$$

$$\text{or, since } 1 - \zeta \text{ equals the filter binding efficiency } \epsilon \quad (\text{eq. A7})$$

$$R = 1 - e^{-\lambda \zeta \tau}$$

$$\text{where } \omega = \frac{K_A P}{1 + K_A P} \quad (\text{eq. A8})$$

In equations A6 and A8, P is the free protein concentration while only the total protein concentration P_0 is precisely known. Since $P_0 = P + P_b$, where P_b is the concentration of protein bound to substrate in solution, and is thus related to R , we can derive the relationship of P to P_0 to obtain an equation which expresses R in terms of known parameters and the association constant K_A . The total molar plasmid concentration is defined as

$$D = \sum_{n=0}^{\infty} D_n \quad (\text{eq. A8})$$

where D_n is the concentration of plasmids containing exactly n dimers. For any single plasmid species containing n dimers and by the assumption of equal and independent binding sites

$$D_n = \sum_{i=0}^n D_{i,n} = D_{0,n} (1 + K_A P)^n = D g_n \quad (\text{eq. A9})$$

where $D_{i,n}$ is the concentration of plasmid molecules containing i photolyase-dimer complexes and n dimers, and g_n is the fraction of plasmids containing exactly n dimers. Following the derivation of Woodbury and von Hippel, if K_i is the phenomenological association constant for the i-th species, then for plasmids containing exactly n dimers the concentration of bound enzyme is

$$(P_b)_n = D_{0,n} \sum_{i=1}^n i K_i P^i \quad (\text{eq. A10})$$

and since for equal and independent binding sites

$$K_i = \frac{n!}{i! (n-i)!} K_A^i \quad (\text{eq. A11})$$

$$\text{then } (P_b)_n = D_{0,n} n K_A P (1 + K_A P)^{n-1} \quad (\text{eq. A12})$$

$$= D g_n \left(\frac{n K_A P}{1 + K_A P} \right) \quad (\text{eq. A13})$$

It follows that the total concentration of bound protein P_b is

$$P_b = \sum_{n=0}^{\infty} (P_b)_n = D \left(\frac{K_A P}{1 + K_A P} \right) \sum_{n=0}^{\infty} n g_n \quad (\text{eq. A14})$$

or,

$$P_b = \lambda D \left(\frac{K_A P}{1 + K_A P} \right) \quad (\text{eq. A15})$$

By definition,

$$P_0 = P + P_b = \lambda D \left(\frac{K_A P}{1 + K_A P} \right) + P \quad (\text{eq. A16})$$

Solving for $K_A P$ we obtain

$$K_A P = \frac{K_A P_0 - (1 + \lambda K_A D)}{2} + \sqrt{\left(\frac{K_A P_0 - (1 + \lambda K_A D)}{2} \right)^2 + K_A P_0} \quad (\text{eq. A17})$$

Substituting back into equation 6 we obtain the final expression for the relationship between τ , D , λ and P (which are known), R (which is determined experimentally) and K_A , the desired association constant

$$R = 1 - e^{-\lambda \zeta \left[\frac{Z}{1 + Z} \right]} \quad (\text{eq. A18})$$

where

$$Z = K_A P = \sigma + \sqrt{\sigma^2 + K_A P_0} \quad (\text{eq. A19})$$

and

$$\sigma = \frac{1}{2} [K_A P_0 - (1 + \lambda K_A D)] \quad (\text{eq. A20})$$

It should be noted that the extent to which the above corrections are necessary depends upon the average number of pyrimidine dimers per molecule (λ) and the value of R. For example with 5.5 dimers per molecule, the difference in K_A values estimated using equations A18-A20 versus equation 6 is < 10% when R < 0.5; however with 2 dimers per molecule, equation A1 underestimates K_A by > 10% at all R values > 0.25.