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Binding of *E. coli* DNA photolyase to a defined substrate containing a single T< >T dimer

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ABSTRACT

The *E. coli* DNA photolyase is a flavoprotein that catalyzes the photoreversal of pyrimidine dimers. The enzyme binds to DNA containing pyrimidine dimers in a light-independent step and repairs the dimer upon absorbing a photon in the 300-600 nm range. The rate and equilibrium constants for the light-independent reaction were determined before, using randomly modified substrates that contained T<>T, T<>C and C<>C dimers in random sequence surrounding. In this paper we have determined these constants for a defined substrate (a 43 bp oligomer containing a T<>T dimer) using the gel retardation assay. We find that: (i) the equilibrium constant and the off rate obtained with this substrate by this technique are similar to those obtained with randomly modified DNA using filter binding and flash photolysis techniques. (ii) the off rate with the defined substrate is heterogenous indicating heterogeneity in the enzyme population or in the enzyme-substrate complexes, and (iii) the enzyme has 7.5 x 10^4-fold higher affinity for pyrimidine dimer compared to non-dimer DNA nucleotides.

INTRODUCTION

DNA photolyase (photoreactivating enzyme) behaves essentially like a classic Michaelis-Menten enzyme (1,2) with the exception that the catalytic step is light-dependent. This dependence has made it possible to study the two steps of the enzymatic reaction (binding and photolysis) individually both *in vivo* and *in vitro*. Flash photolysis was utilized in an ingenious set of experiments to measure the association and dissociation rate constants (k_1 and k_2) as well as the equilibrium binding constant, K_a, *in vivo* (3). More recently these parameters of the dark reaction were measured *in vitro* by both the nitrocellulose filter binding assay as well as flash photolysis and transformation, using UV irradiated pBR322 as substrate (4). The *in vivo* and *in vitro* values agreed reasonably well. Both studies showed that the two first-order rate constants, the light independent k_2 as well as the light-dependent rate coefficient k_3 were composites made up of at least two components. It was suggested that this heterogeneity in the rate coefficients was caused by the different types of pyrimidine dimers as well as

by the effects of neighboring sequences on the ES complex formed with a given type of a dimer (3,4,5). In the present study we have investigated the binding of photolyase to a unique substrate, a 43-bp duplex that contains a thymine dimer in a central location. Using this unique substrate and the gel retardation assay (6,7) we have measured the K_a and k_2 for binding of photolyase to thymine dimer. We find that both the thermodynamic and kinetic constants for this substrate are similar to those obtained with heterogeneous substrates. Surprisingly, we also find that k_2 is heterogeneous even with the defined substrate suggesting that the multicomponent nature of this coefficient is due to an intrinsic heterogeneity in the enzyme molecules or to the formation of heterogeneous complexes between two homogeneous reactants. In addition the gel retardation assay has enabled us to measure the affinity of photolyase to nonsubstrate DNA which is approximately 7.5 x 10^4-fold lower than that for pyrimidine dimers.

MATERIALS AND METHODS

Enzyme. *E. coli* DNA photolyase was purified as described previously (8) and was better than 98% pure as analyzed by SDS-polyacrylamide gel electrophoresis. The enzyme preparations used were free of endo- or exonucleases under the assay conditions used.

Substrate. The substrate was a 5' labeled 43-bp duplex that was constructed by ligating an 11-mer containing a *cis-syn* thymine dimer (kindly provided by Dr. Christopher W. Lawrence of University of Rochester) with 5 other oligomers. The construction and characterization of this substrate will be described elsewhere. The double stranded non-substrate competitive DNA was pBR322 superhelical DNA. DNA concentrations were measured by absorbance at 260 nm. M13mp8 phage DNA was used as the single stranded non substrate DNA. For k_2 measurements UV-irradiated pBR322 was used as the competitive substrate.

Gel Retardation assay. The reaction mixture contained Tris.HCl 50 mM, pH 7.5, NaCl 100 mM, β-mercaptoethanol 1.0 mM, EDTA 1 mM, bovine serum albumin 100 μg/ml and, enzyme and substrate at the indicated amounts. The mixture was incubated at 23°C for 45 minutes, glycerol was added to a final concentration of 6% and loaded onto an acrylamide gel and subjected to electrophoresis. To follow the migration xylene cyanol and bromophenol blue were added to a separate lane. The polyacrylamide gel (5%) contained acrylamide/methylenebisacrylamide at 30:1 ratio in Tris borate 100 mM, EDTA 1 mM, pH 8.3. The gel was prerun for 10 min before loading the samples, in

the equilibrium experiments. In the kinetic experiments samples were loaded onto the running gel. The gel was 20 cm-long, 16 cm wide and 0.3 cm thick. Electrophoresis was carried out at constant current of 30 mA until the bromophenol blue had migrated approximately 13 cm. The gel was then autoradiographed to locate the free DNA and the ES complexes. The bands were excised and the amount of bound DNA was determined by Cerenkov counting of the free and retarded DNA. The amount of ES complexes was calculated in two ways: In one method the radioactivity associated with the fast migrating band as a fraction of the total DNA loaded onto the gel was taken to be a measure of the unbound DNA and the rest of the DNA was assumed to be enzyme bound. Alternatively the DNA associated with the slow migrating band was taken as a measure of the ES complex. The first method includes the "streaking" DNA into the bound category and therefore gives a higher value for the bound fraction and may be considered a better method for obtaining the true value of the ES complexes. However, that method is subject to inaccuracies caused by sampling errors as it assumes that all samples contained the same amount of DNA as the control sample.

Filter Binding Assay. This assay as applied to photolyase has been described in detail elsewhere (4,9,10). Briefly a 50-µl reaction mixture was filtered through a 24 mm nitrocellulose filter (Schleicher-Schull), the filter was washed 3 times with 200 µl of reaction buffer minus bovine serum albumin and the filters were dried and DNA retained on filters was quantitated by Cerenkov counting.

RESULTS

Gel Retardation Assay with *E. coli* Photolyase. The gel retardation assay has been used extensively for determining the thermodynamic and kinetic parameters of a number of DNA binding proteins as well as in identification and isolation of proteins that bind to specific DNA sequences (11). However, there are no general buffer-gel systems applicable to all protein-DNA complexes and for each protein the optimum conditions for obtaining a DNA-protein band are determined empirically. For *E. coli* photolyase we found the gel buffer system described in he Materials and Methods section adequate for our purposes. We used the DNA fragment shown in Figure 1 as substrate. This 43 bp-long fragment contains a thymine dimer at a central location. In Figure 2 we compare the binding of photolyase to this substrate as well as to its non-dimer containing couterpart. The enzyme binds to the dimer containing DNA specifically and increasing the amount of enzyme

5′CTATCGATGGCCTGCAGGCAAGTΛTGGAGGAATTCGTACTGAGTC 3′
3′ ATAGCTACCGGACGTCCGTTCAACCTCCTTAAGCATGACTCAGT5′

Figure 1. Sequence of the synthetic substrate used in this study. The location of the dimer is shown by Λ.

results in the increase in the fraction of DNA migrating with the enzyme band. Large amount of non-substrate DNA decreases the fraction of the specific complexes formed indicating a weak interaction with non-substrate DNA. However, when we attempted to detect the non-specific binding to non-dimer containing fragments by the appearance of an ES band similar to that seen with the substrate we failed to observe any such band even at very high enzyme concentration. This is presumably because the enzyme-non substrate complexes are too weak and do not survive the electrophoresis. A similar phenomenon was observed with the nitrocellulose filter binding assay (4,10).

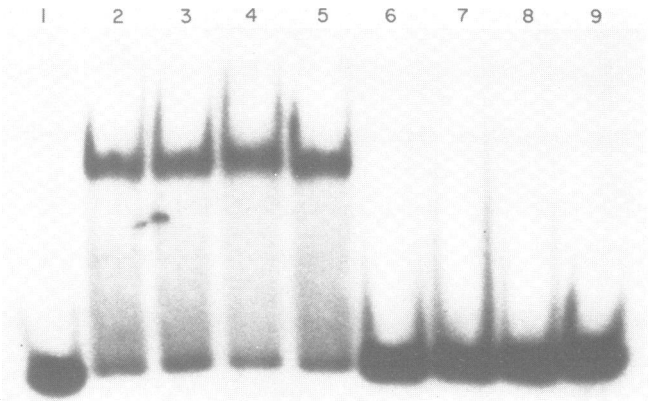

Figure 2. Binding of photolyase to a DNA fragment with and without a thymine dimer. Photolyase was incubated for 45 min at 23°C with 43-bp fragment (labeled at the 5' end of the non-dimer strand) in 50 µl reaction mixtures containing ~ 0.50 pmol of labeled DNA in Tris-HCl 50 mM, NaCl 100 mM, EDTA 1 mM, 2-mercaptoethanol 1 mM, bovine serum albumin 100 μ_g/ml and the indicated amounts of enzyme and non-irradiated pBR322 DNA. After incubation the samples were made 6% in glycerol and loaded on a 5% polyacrylamide gel which was prerun at 120 volts for 15 min. After electrophoresis and autoradiography the photolyase-bound and free DNA bands were cut out and the radioactivity in these bands was measured by Cerenkov counting. Each lane in addition to labeled DNA, contained the following: Lane 1, substrate with no addition; 2-4, substrate plus 92, 166 and 962 nM of photolyase, respectively; 5, substrate fragment plus 962 nM photolyase and 52 µM (bp) of pBR322 DNA; 6-7 non-substrate fragment with 92 and 962 nM of photolyase, respectively; 8, non-substrate fragment plus 962 nM photolyase and 52 µM (bp) of pBR322; Lane 9 contains non-substrate DNA with no additions.

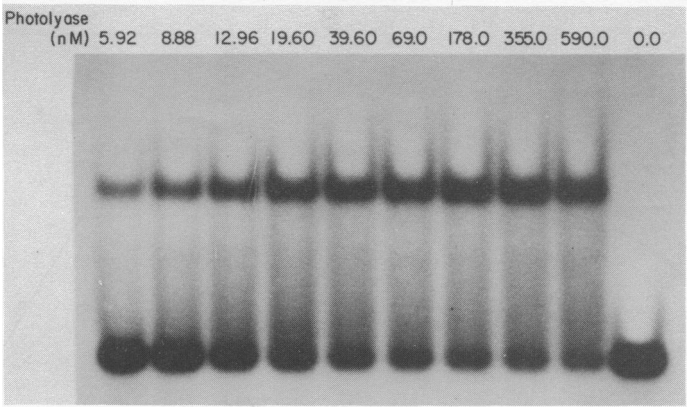

Figure 3. Concentration dependent formation of ES complexes as measured on a polyacrylamide gel. The substrate at 10 nM was incubated with the indicated concentrations of photolyase for 45 min at 23°C and then subjected to electrophoresis and autoradiography.

Specific Binding Constant. The K_a for formation of photolyase-thymine dimer complex was determined by incubating increasing amounts of enzyme with a constant amount of substrate and quantitating the fraction of enzyme-bound DNA at each concentration. The result of such an experiment is shown in Figure 3. The data points from this experiment and two other runs under identical conditions were plotted to obtain the "saturation curve" shown in Figure 4A. Analysis of this curve by Scatchard plot gives the results shown in Figure 4B. From the Scatchard plot we obtain a value of $K_a = 2.6 \times 10^8$ M^{-1} for binding of photolyase to a thymine dimer and a stoichiometry of 1.0 molecule per dimer in a reasonable agreement with the Ka values determined by filter binding or flash photolysis techniques. For a direct comparison of the gel retardation and filter binding assays we ran half of the reaction mixture on a retardation gel and filtered the other half. To our surprise we found that only 25% percent of the substrate was retained on the filters at saturating enzyme concentrations. We had previously found that with pBR322 DNA as a substrate 34% of the specific complexes were retained on the filter. We have no satisfactory explanation for the discrepancy. However, assuming that the retention efficiency of the ES complexes on nitrocellulose filters is 25%, the amount of ES complexes at each enzyme concentration can be calculated and compared with the values obtained from the gel retardation assay. As is seen in Figure 4 when this correction is made there is a perfect agreement between the results obtained by these two methods.

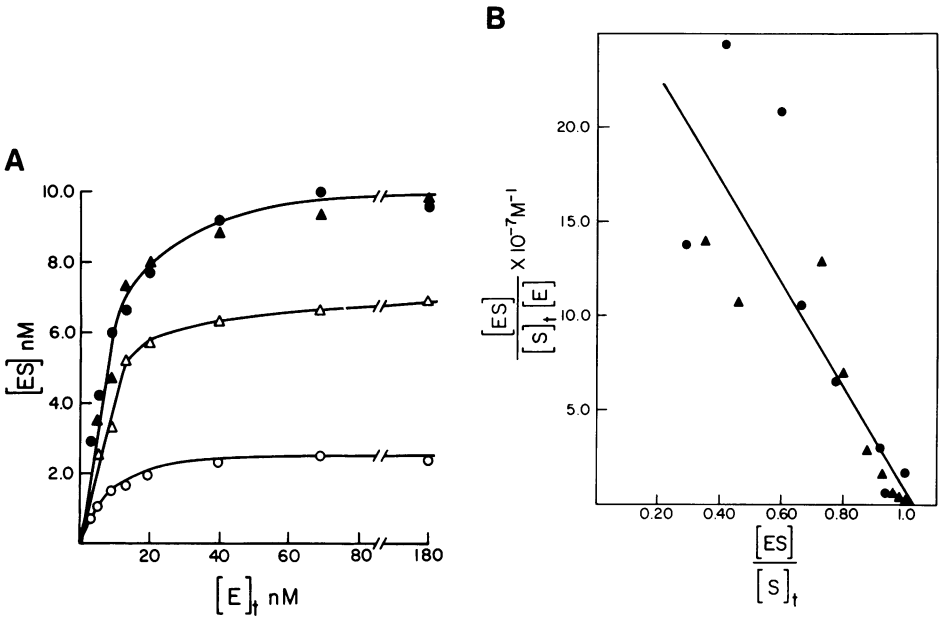

Figure 4. Determination of the equilibrium constant by the gel retardation and filter binding methods. The substrate was incubated at 10 nM concentration with increasing amounts of photolyase in a 100 µl reaction mixture as described in Figure 1. Half of the reaction mixtures were filtered and the other half were electrophoresed. The amount of ES complexes were determined by quantitating the radioactivity retained on the filters and the radioactivity migrating at the location of "free DNA" on the polyacrylamide gel. The open symbols represent the actual experimental data points while the closed symbols represent normalized [ES] values assuming the stability of the ES complexes (efficiency of retention) is 0.25 for the nitrocellulose filters and 0.65 for the polyacrylamide gel. Circles, nitrocellulose filtration; triangles, gel retardation. (A) The binding data; (B) Analysis of the binding data by Scatchard plot assuming that at 180 nM photolyase all the substrate was in complexed form. The data points are the average values from 3 experiments.

Non-specific Binding Constant. The binding of photolyase to non-damaged DNA cannot be measured directly by either the filter binding assay or gel retardation because presumably the complexes are too transient and do not survive the experimental probe. We therefore determined the affinity of the enzyme to non-substrate DNA indirectly, by measuring inhibition of the specific complex formation. The results of such an experiment are shown in Figure 5. At substrate concentration of 1×10^{-8} M and enzyme concentration of 1×10^{-8} M, non-substrate DNA concentration of 3.75×10^{-4} M (in nucleotides) reduced the fraction of ES complexes to one half. From these values

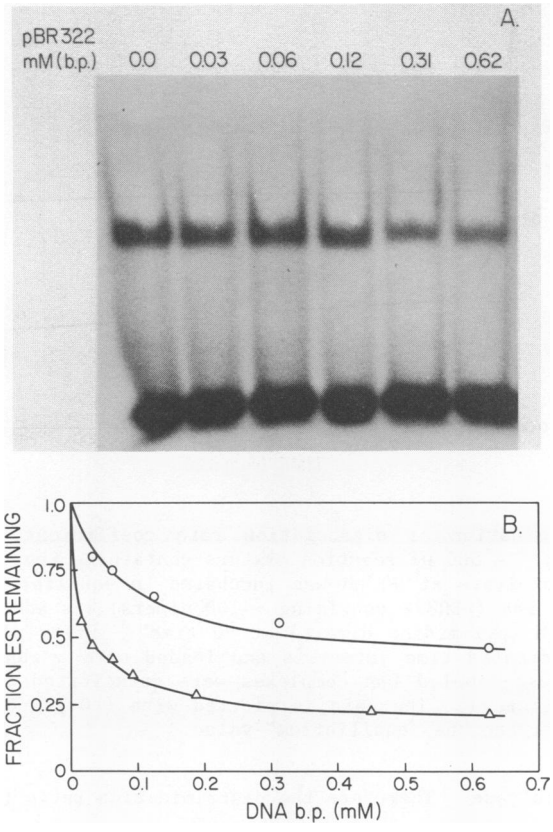

Figure 5. Determination of the association constant of photolyase and undamaged DNA. The 43 bp radiolabeled substrate at 10 nM was mixed with the indicated amounts of undamaged pBR322 or M13 ss DNA and 10 nM of photolyase, incubated at 23°C for 45 min and then subjected to electrophoresis and autoradiography. The free and enzyme-bound DNA were quantitated by Cerenkov counting. In panel A we show a photograph of the polyacrylamide gel when pBR322 was the competing substrate. The first lane contained DNA only, the other lanes contained photolyase as well as pBR322 at the indicated concentrations. Panel B is a plot of the specific ES complexes as a function of increasing non-specific DNAs. 0, pBR322 double strand DNA; Δ, M13 single strand DNA.

we calculate the affinity of the enzyme to non-substrate DNA from the following equation (12).

$$K_N = \frac{K_S\ [S]}{2D_{\frac{1}{2}}}$$

$D_{\frac{1}{2}}$ = non-specific DNA concentration that reduces the specific complexes by one-half; K_S = specific binding constant, and K_N = non-specific binding

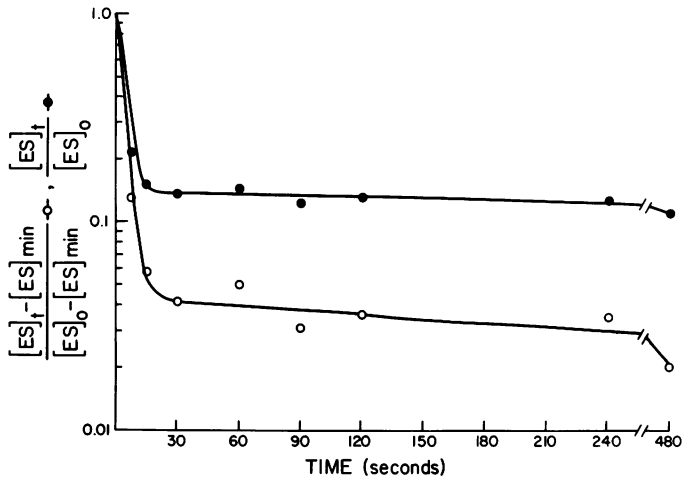

Figure 6. Determination of dissociation rate coefficient k_2 by the gel retardation assay. A 500 µl reaction mixture containing the 43 bp substrate at 10 nM and photolyase at 90 nM was incubated to equilibrium (45 min) at 23°C. Competing DNA (pBR322 contining ~ 100 dimers) was added at a concentration of 285 nM (pyrimidine dimers) at "0 time". Fifty µl aliquots were taken at the indicated time intervals and loaded onto a running gel. The specific photolyase-labeled DNA complexes were quantitated as described in the legend of Figure 1. The data is plotted with (-O-) and without (-●-) the correction for the "new equilibrium" value.

constant for photolyase. Therefore the discrimination ratio (or selectivity) for DNA photolyase is $K_S/K_N = 7.50 \times 10^4$. This is in agreement with the selectivity of the enzyme that was predicted from *in vivo* flash photoreactivation experiments (3,4). When the same experiment was repeated with single stranded DNA as the competitor we found $D_{\frac{1}{2}} = 3.0 \times 10^{-5}$M indicating that the enzyme has higher affinity to single stranded than double stranded DNA.

The Dissociation Rate Coefficient. This rate coefficient was determined by adding excess unlabeled substrate (UV irradiated pBR322) to a preequilibrated enzyme-labeled substrate complex and measuring the decay of the preexisting complexes as a function of time by taking samples from the reaction mixture and loading unto a running gel (13). The results of such an experiment are shown in Figure 6. We have plotted this first-order graph in two ways. First we assumed that competition was 100% efficient and therefore the decay of the ES complexes was representative of the actual kinetics. However, even after extensive incubation with the competing substrate equilibrium was not reached, about 10% of the initial complexes

TABLE 1. Reaction Constants for Binding of Photolyase to DNA.

Method	Ka (M^{-1})	k_1 ($M^{-1}s^{-1}$)	k_2(s^{-1})
Flash photolysis (4)	4.7×10^{7}	$1.4\text{-}4 \times 10^{6}$	3×10^{-2} (fast) 6×10^{-4} (slow)
Filter binding (4)	6×10^{7}	ND	ND
Gel retardation	2.6×10^{8}	ND	4.4×10^{-2} (fast) 3.55×10^{-4} (slow)
Kinetic (k_1/k_2) (4)	$0.47\text{-}1.4 \times 10^{8}$	NA	NA
In vivo (flash) (3)	1×10^{8}	1.1×10^{6}	1.3×10^{-2} (fast) 6×10^{-4} (slow)

ND, not determined; NA, not applicable.

remained after 1 hr of incubation (even though the fraction of dimers in the radioactive DNA was only 3% of total dimers). Therefore if one assumes that this fraction of the complexes do not contribute to the kinetics then a new first order decay kinetics is obtained where from each time point the final equilibrium [ES] value has been subtracted. In Figure 6 the data have been plotted both ways. The k_2 values obtained from this figure are within the range of k_2 values we reported previously using the flash photolysis method (Table 1). However, to our surprise we find that the dissociation kinetics with this defined substrate is similar to that obtained with heterogeneous substrates in being composed of at least two components. We calculate k_2 = $4.44 \pm 2.3 \times 10^{-2}$ ($5.85 \pm 3.75 \times 10^{-2}$) s^{-1} and k_2 = $3.55 \pm 1.49 \times 10^{-4}$ ($8.15 \pm 2.3 \times 10^{-4}$) s^{-1} for the fast (85-95%) and slow (5-15%) components, respectively. The k_2 values in parentheses are those obtained after subtracting the equilibrium values. The fast off rates obtained by this method are about 2-fold higher than those obtained with the flash photolysis method. We believe that this difference is due to the inherent problems of the gel retardation assay (i.e. in the time required for the ES complexes loaded into the wells to reach the gel matrix some dissociation occurs) and that the flash photolysis values are closer to the real k_2 values. Nevertheless the gel retardation assay reveals an important fact: even with a given type of dimer in a defined sequence the dissociation of photolyase from its substrate is not a simple first order reaction for all the complexes.

DISCUSSION

We have used the gel retardation assay to investigate the specific binding of photolyase to a defined substrate as well as to non-substrate

DNA. We find that the enzyme binds to a T<>T dimer with an association constant K_a = 2.6 x 10^8 M^{-1} as measured by both the gel retardation and nitrocellulose filter binding assays. This value is 4-5-fold higher than the values we reported earlier which were obtained by both the nitrocellulose filter binding and flash photolysis assays using UV-irradiated pBR322 (~ 5 pyrimidine dimer per molecule). We do not know the exact cause of this discrepancy, however, we do not think that the type of dimers is responsible for the difference. At a frequency of ~ 1 dimer/kbp of *E. coli* DNA about 90% of the dimers are thymine dimers (14) and therefore the binding of the enzyme to UV-irradiated pBR322, most likely is dominated by the interaction of the enzyme with thymine dimers. The discrepancy may be partly explained by the fact that with UV-pBR322 substrate the dimers are embedded in a 10^4-fold excess of non-substrate DNA whereas with the unique substrate the ratio of non-substrate nucleotides to substrate is 44. Nevertheless the affinity of the enzyme to non-substrate DNA is not large enough to account for the difference completely. Perhaps another contributing factor is that we have normalized the maximum binding observed with the defined oligomer to 100% both in the gel retardation and filter binding assays and such normalization is only partly justified as (especially with the gel retardation assay) there is a slow but steady increase in the fraction of bound DNA even at the highest enzyme concentrations used in our experiments. Taking all these factors into account we believe that Ka = 10^8 M^{-1} is the most accurate value for the binding of photolyase to pyrimidine dimers.

Von Hippel and Berg (15) have recently discussed the various factors that must be considered in describing the binding of a protein to a specific DNA structure. Although these authors discussed the aspects of "sequence-specific" binding, their generalizations are equally applicable to "structure-specific" binding proteins as well. The authors propose that in order to define specific DNA-protein interactions with precision various levels of specificity must be defined: specification (the length-in base pairs of the sequence involved in specifying the target binding site), recognition (the physico-chemical mechanisms that control the specific interactions), discrimination or selectivity (the differences in the affinity of the protein for the various DNA targets, and finally selection (the effective binding relation for the whole system of protein and DNA binding sites). In terms of this nomenclature an oligodT_3 with a pyrimidine dimer specifies the binding of photolyase (16) which recognizes substrate mainly through non-ionic interactions (4) and has a discrimination ratio (or selectivity

factor) of $K_S/K_N = 7.5 \times 10^4$. This selectivity is achieved by the unusually low binding constant ($K_N = 3.47 \times 10^3\ M^{-1}$) of photolyase to non-substrate DNA. Thus the enzyme is able to select the 10-20 dimers in an *E. coli* cell and repair them instantaneously (17) despite the fact that an *E. coli* cell contains only 10-20 photolyase molecules and 10^7 DNA nucleotides (each of which may be considered the beginning of a potential binding site).

Both of the first order rate constants k_2 and k_3 in the reaction scheme for photolyase

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[h\nu\ (300\text{-}600\ nm)]{k_3 = k_p I} E + P$$

were previously found to be multicomponent with natural DNA substrate (3,4) and it was suggested that this heterogeneity was due to a combination of heterogeneity in the types of dimers as well as in the sequences surrounding dimers. It was therefore quite unexpected that k_2 of photolyase-thymine dimer at a defined sequence should also be biphasic. We find that about 85% of the complexes dissociate rapidly with a half-life of ~ 15-45 sec while the rest dissociate with a half-life of ~ 30 min. Thus, two apparently homogeneous components (photolyase and the dimer containing 43-mer) seem to make at least two different classes of complexes or the two components make a homogeneous complex that dissociate by two separate pathways. (It is debatable whether these two models are alternative explanations for the kinetic process or alternative statements for the same physical phenomenon.) It is known that the FAD co-factor of the purified photolyase is in neutral radical form which can be oxidized or reduced to FAD_{ox} or $FADH_2$, respectively (18). The oxidation of flavin changes the binding properties of the enzyme (4) and therefore it is conceivable that the biphasic dissociation kinetics reflect the dissociation of the blue (major) and yellow (minor) forms of the enzyme. This may be a contributing factor but cannot account for the magnitude of the fraction of the slow component. Fluorescence measurement with the enzyme preparation used in these studies shows that less than 10% (the limit of our resolution) of FAD associated with the enzyme is oxidized. Another potential source of heterogeneity may be the second chromophore (16) content of the enzyme. However we have determined (G. Payne, I. Husain, and A. Sancar, unpublished observation) that enzyme devoid of second chromophore has the same dissociation kinetics (two components) as the native enzyme. We, therefore conclude that other factors such as "partially unfolded enzyme" or substrate in non-B form may contribute to the complex dissociation kinetics.

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