Reaction of *Escherichia coli* and Yeast Photolyases with Homogeneous Short-Chain Oligonucleotide Substrates†

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ABSTRACT: Similar rates have been observed for dimer repair with *Escherichia coli* photolyase and the heterogeneous mixtures generated by UV irradiation of oligothymidylates [UV-oligo(dT)$_n$, $n \geq 4$] or DNA. Comparable stability was observed for ES complexes formed with UV-oligo(dT)$_n$ ($n \geq 9$) or dimer-containing DNA. In this paper, binding studies with *E. coli* photolyase and a series of homogeneous oligonucleotide substrates (TpT, TpTp, pTpT, TpTpT, TpTpT, TpTpTpT, TpTpTpT, TpTpTpT, TpTpTpT) show that about 80% of the binding energy observed with DNA as substrate ($\Delta G \sim 10$ kcal/mol) can be attributed to the interaction of the enzyme with a dimer-containing region that spans only four nucleotides in length. This major binding determinant (TpTpTpT) coincides with the major conformational impact region of the dimer and reflects contributions from the dimer itself (TpT, $\Delta G = 4.6$ kcal/mol), adjacent phosphates (5′p, 0.8 kcal/mol; 3′p, 1.1 kcal/mol), and adjacent thymine residues (5′T, 0.8 kcal/mol; 3′T, 1.3 kcal/mol). Similar turnover rates (average $k_{cat} = 6.7$ min^{-1}) are observed with short-chain oligonucleotide substrates and UV-oligo(dT)$_{18}$, despite a 25 000-fold variation in binding constants (K_d). In contrast, the ratio K_m/K_d decreases as binding affinity decreases and appears to plateau at a value near 1. Turnover with oligonucleotide substrates occurs at a rate similar to that estimated for the photochemical step (5.1 min^{-1}), suggesting that this step is rate determining. Under these conditions, K_m will approach K_d when the rate of ES complex dissociation exceeds k_{cat}. This restriction is compatible with calculations which indicate that weaker binding with oligonucleotide substrates is due to slower formation and faster dissociation of the ES complex. Yeast photolyase exhibits a near absolute requirement for phosphate immediately 3′ to the dimer. However, turnover rates with all other isomers tested ($k_{cat} = 4.8$ min^{-1}) are similar to that observed with *E. coli* photolyase. Variations in K_m values observed with yeast photolyase suggest that the major binding determinant (TpTpTpT) is different from that observed for *E. coli* photolyase.

The principle damage resulting from exposure of DNA to ultraviolet light is the formation of *cis-syn*-cyclobutane dimers between adjacent pyrimidine residues. The presence of UV-induced lesions in DNA has been associated with mutagenesis, carcinogenesis, and cell death. Cyclobutane dimers can be monomerized by DNA photolyase in an unusual enzymatic reaction which requires visible light. All known DNA photorepair enzymes contain two chromophores which act as photosensitizers in catalysis. One of the chromophores is always 1,5-dihydro-FAD ($FADH_2$).[1] In the case of *Escherichia coli* photolyase, oxidation of $FADH_2$ to a blue neutral radical ($FADH^•$) occurs during enzyme isolation, but the process can be reversed with dithionite or by photoreduction (Jorns et al., 1987a,b; Sancar et al., 1987a). The known DNA photolyases can be divided into two classes, depending on the nature of the variable second chromophore [see recent review by Jorns (1989)]. *E. coli* and yeast photolyases belong to the same class and contain 5,10-methenyltetrahydropteroylpolyglutamate as a second chromophore (Wang et al., 1988; Johnson et al., 1988; Wang & Jorns, 1989).

Our previous studies with UV-irradiated oligothymidylates [UV-oligo(dT)$_n$] showed that these simple, single-stranded DNA analogues are good substrates for *E. coli* photolyase (Jorns et al., 1985). That the enzyme interacts with a rather limited region in DNA near the dimer was suggested, in part, by the fact that a single molecule of UV-oligo(dT)$_{18}$ (3.5 dimers/oligomer) could tightly bind two molecules of photolyase. In addition, similar binding affinity was observed with UV-irradiated oligomers containing nine or more thymine residues and with double-stranded plasmid DNA (Jordan & Jorns, 1988). Studies with *E. coli* photolyase and UV-irradiated oligonucleotides suggested that major binding determinants might be localized in a small region immediately surrounding the dimer (Jordan & Jorns, 1988), but further analysis was limited by substrate heterogeneity since UV irradiation converts a pure oligothymidylate preparation into a complex mixture of isomers, particularly when multiple dimers are introduced. In this paper we show that homogeneous short-chain oligonucleotide substrates can be used to obtain a detailed picture of binding determinants and also to identify the rate-determining step in catalysis by *E. coli* photolyase. Comparison studies show that yeast photolyase can also repair dimers in very small DNA analogues (e.g., TpTp) but that the eukaryotic and prokaryotic enzymes exhibit significant differences in binding determinants.

Experimental Procedures

Materials. Oligothymidylates (pT)$_n$ were obtained from P-L Biochemicals ($n = 4, 6, 9, 18$) or from Sigma Chemical

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^•$, blue neutral FAD radical; $FADH_2$, 1,5-dihydro-FAD; DTT, dithiothreitol; 5-CH_3-H_4Pte(Glu)$_n$, 5-methyltetrahydropteroylpolyglutamate; UV-oligo(dT)$_n$, dimer-containing oligothymidylate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Table I: Reaction of *E. coli* Photolyase with Various Substrates: Thermodynamic and Kinetic Parameters

	ES complex formation		steady-state parameters		k_1[a] (M^{-1} min^{-1} × 10^{-4})		k_{-1}[a] (min^{-1})	
substrate	K_d (μM)	ΔG (kcal/mol)	K_m (μM)	k_{cat} (min^{-1})	calcd	min value	calcd	min value
TpT	250	4.6	220	5.3		2.4		6.0
pTpT	55	5.4	42	6.0		14		7.9
TpTp[b]	32	5.7	38	7.0		18		5.9
TpTpT	14	6.2	68	7.2	12	11	1.7	1.5
TpTpTpT	8.2	6.5	49	6.4	16	13	1.3	1.1
TpTpTpTpT	3.5	6.9	21	6.6	38	31	1.3	1.1
TpTpTpT	2.9	7.0	22	6.6	35	30	1.0	0.87
TpTpTpTpT	0.37	8.2	8.0	8.8	91	110	0.34	0.41
TpTpTpTpT	2.9	7.0	17	7.2				
UV-oligo$(dT)_{18}$	≤0.01[c]	≥10	*d*	6.8				

[a] Rate constants for the formation (k_1) and the dissociation (k_{-1}) of the ES complex were calculated and minimum values were estimated as described under Discussion. [b] Values were obtained with substrate prepared by the method of Jorns et al. (1985). Similar results (K_d = 45 μM, K_m = 32 μM, k_{cat} = 5.5 min^{-1}) were obtained with substrate prepared according to the method of Rycyna et al. (1988). [c] Value taken from Jordan and Jorns (1988). [d] K_m is too small to measure with the current assay.

Co. (n = 2). TpTp was a generous gift from Dr. John Gerlt.

Enzymes. Unless otherwise specified, all handling of enzymes was done under yellow light. The blue form of *E. coli* photolyase was purified as previously described (Jorns et al., 1987a). The concentration of the isolated *E. coli* enzyme was calculated on the basis of its absorbance at 580 nm [ϵ_{580} = 4.8 × 10^3 M^{-1} cm^{-1} (Wang & Jorns, 1989)]. Yeast photolyase was purified according to a procedure similar to that described by Sancar et al. (1987b). The concentration of the yeast enzyme was calculated on the basis of its absorbance at 278 nm [ϵ_{278} = 12.8 × 10^4 M^{-1} cm^{-1} (Sancar et al., 1987b)].

Substrates. Oligothymidylates containing cis-syn dimers at specific sites (TpT, TpTp, TpTpT, TpTpT, TpTpTpT, TpTpTpT, TpTpTpT, TpTpTpT) were synthesized and purified as described by Rycyna et al. (1988). Whenever possible, oligomer concentrations were determined on the basis of the absorbance of monomeric thymine residues at 260 nm [ϵ_{260} = 8.3 × 10^3 M^{-1} cm^{-1} (Naylor & Gilham, 1966; Ts'o et al., 1966; Jorns et al., 1985)]. In other cases, oligomer concentrations were calculated on the basis of the absorbance of the dimer at 220 nm [ϵ_{220} = 4.0 × 10^3 M^{-1} cm^{-1} (Herbert et al., 1969)]. All other substrates were prepared by irradiating commercially available oligothymidylates or TpTp with ultraviolet light as described by Jorns et al. (1985). The dimer content of the irradiated samples was determined as previously described (Jorns et al., 1985).

Binding Studies. Binding of oligonucleotide substrates to *E. coli* photolyase was measured according to the fluorescence titration method described by Jordan and Jorns (1988). In this procedure, the isolated enzyme is treated with sodium borohydride, which reduces the pterin chromophore, followed by reduction with dithionite, which converts the flavin radical to $FADH_2$. Reduction of the pterin chromophore eliminates the chromophore's fluorescence but does not affect catalytic activity or the ability of substrate to quench $FADH_2$ fluorescence, which is monitored at 540 nm (excitation λ = 390 nm). Titrations were typically performed in 50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol (complete PRE buffer) at 5 °C because optimal enzyme stability was achieved under these conditions. However, similar values for K_d were observed in studies with pTpT in complete PRE buffer at 5 °C (K_d = 55 μM), in assay buffer (50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1.7 mM DTT, and 1 mM EDTA) at 5 °C (K_d = 57 μM), or in complete PRE buffer at 21 °C (K_d = 54 μM). Binding constants for weakly bound substrates were determined by analyzing the data according to the method of Benesi and Hildebrand (1949) or by using an end point obtained by adding an aliquot of UV-oligo$(dT)_{18}$ at the end of the titration.

Steady-State Kinetics. Dimer repair with oligothymidylate substrates was measured according to the method described by Jorns et al. (1985) by following the increase in absorbance at 260 nm which accompanies the monomerization of thymine dimers. Enzyme assays with yeast photolyase were performed at 21 °C in 50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl and 1 mM EDTA. The same conditions were used with *E. coli* photolyase, except that 1.7 mM DTT was added to the assay buffer. In the presence of DTT, the flavin radical in the isolated *E. coli* enzyme is rapidly photoreduced under assay conditions, and reaction rates are identical with those observed in assays initiated with enzyme containing $FADH_2$ (Jorns et al., 1987a). Photoreduction is unnecessary with yeast photolyase which is isolated in the $FADH_2$ form (Sancar et al., 1987b). Values for K_m and k_{cat} were calculated by linear least-squares analysis.

Results

ES Complex Formation with E. coli Photolyase. Our previous studies (Jordan & Jorns, 1988) have shown that formation of an enzyme–substrate complex with dimer-containing oligothymidylates results in the selective quenching of the fluorescence due to $FADH_2$ bound to *E. coli* photolyase and that the dissociation constant of the ES complex can be determined by fluorometric titration. (Dimer-free oligothymidylates do not quench $FADH_2$ fluorescence.) We have used this approach to examine the interaction of the enzyme with each of the positional isomers generated by the introduction of a single dimer into an oligothymidylate containing two, three, or four thymine residues. In the case of the 4-mer, we have also studied an isomer containing two dimers (TpTpTpT). Figure 1 shows fluorometric titration data obtained with TpTpTpT. A similar quenching of $FADH_2$ fluorescence was observed with all substrates tested. In each case, the observed quenching of $FADH_2$ fluorescence was fully reversible upon exposure of the ES complex to photoreactivating light, similar to that observed in previous studies with heterogeneous substrates (Jordan & Jorns, 1988). The dissociation constants determined for the various ES complexes varied over nearly 3 orders of magnitude (Table I). The tightest binding was observed with the 4-mer that contained a dimer between the middle two thymine residues (TpTpTpT, K_d = 0.37 μM) whereas the weakest binding was seen with TpT (K_d = 250 μM). Results obtained in titration studies with TpTpT were unaffected by the presence of a 10-fold excess of pTpTpT, consistent with negligible binding of the dimer-free oligomer.

The contribution of the various portions of the TpTpTpT molecule to its overall binding energy can be estimated on the

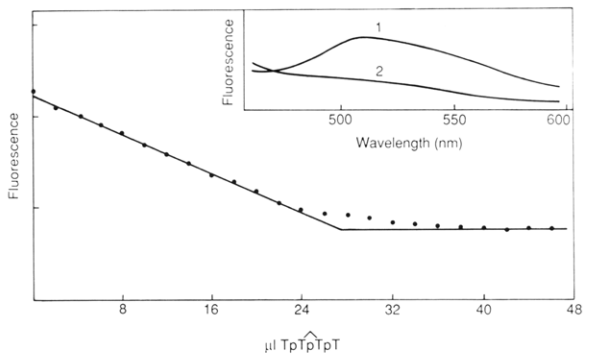

FIGURE 1: Titration of *E. coli* DNA photolyase with TpTp̂TpT. The fluorescence of the pterin chromophore in the isolated enzyme was eliminated by reduction with borohydride, and then dithionite was added to reduce the nonfluorescent flavin radical to $FADH_2$, as described under Experimental Procedures. The quenching of $FADH_2$ fluorescence was monitored at 540 nm (excitation at 390 nm). Inset: Curve 1 is the fluorescence emission spectrum of the enzyme (10.7 μM with respect to $FADH_2$) after reaction with borohydride and dithionite. Curve 2 was recorded at the end of the titration after addition of 38.7 μM TpTp̂TpT (46 μL). The fluorescence emission spectrum observed for borohydride-treated enzyme, before reduction with dithionite, superimposed with curve 2.

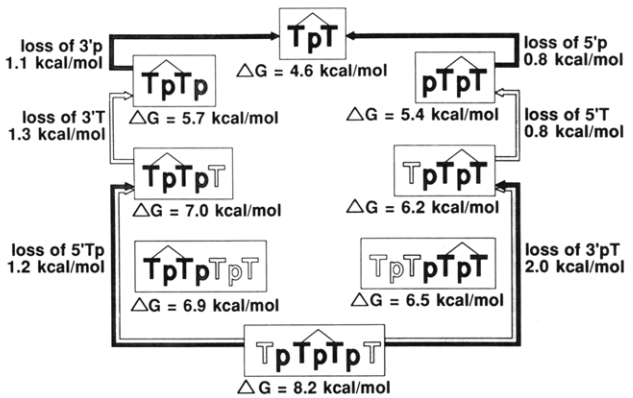

FIGURE 2: Thermodynamic relationships between enzyme–substrate complexes formed with *E. coli* photolyase and various short-chain oligonucleotide substrates. The contribution of the dimer and residues near the dimer to total binding energy was estimated on the basis of differences in ΔG values observed for ES complex dissociation.

basis of results obtained with other isomers. Thermodynamic relationships are most easily visualized when the isomers are arranged as shown in Figure 2. The difference in binding energy observed with TpTp̂T versus TpTp or pTpTp̂T can be used to estimate the energy due to the interaction of the enzyme with a phosphate residue immediately 3′ (ΔG = 1.1 kcal/mol) or 5′ (ΔG = 0.8 kcal/mol) to the dimer. The estimated values are similar to a value (1.1 kcal/mol) calculated by Record et al. (1976) for the electrostatic interaction of a positively charged lysine residue with a phosphate at a salt concentration similar to that used in our binding studies. The energy due to the interaction of photolyase with a thymine residue immediately 3′ (ΔG = 1.3 kcal/mol) or 5′ (ΔG = 0.8 kcal/mol) to the dimer can be estimated on the basis of the difference in binding energy observed with TpTp̂Tp versus TpTp̂TpT or pTpTp̂T versus TpTpTp̂T, respectively. The preceding analysis can be used to predict interaction energies for a phosphate plus thymine combination immediately 3′ (2.4 kcal/mol) or 5′ (1.6 kcal/mol) to the dimer. These values are in reasonable agreement with interaction energies estimated for a 3′pT (ΔG = 2.0 kcal/mol) or a 5′Tp (ΔG = 1.2 kcal/mol) on the basis of the difference in binding energy observed for TpTp̂TpT versus TpTpTp̂T or TpTp̂TpT, respectively. Overall, the results indicate that interaction energetics are somewhat more favorable for groups located immediately 3′ to the dimer as compared with equivalent groups on the 5′ side. The ΔG observed for the dissociation of the enzyme–TpTp̂TpT complex (8.2 kcal/mol) falls within the range (7.6–8.4 kcal/mol) calculated from results obtained with TpTp̂T (ΔG = 4.6 kcal/mol) plus interaction energies estimated for a 3′pT (2.0–2.4 kcal/mol) and a 5′Tp (1.2–1.6 kcal/mol).

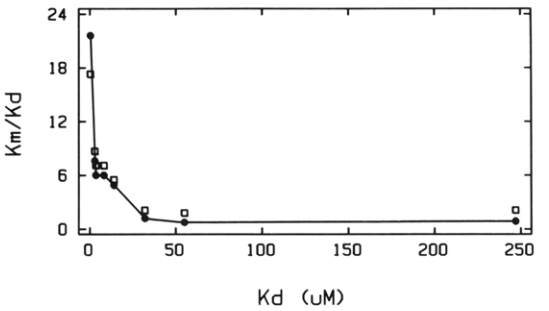

FIGURE 3: Relationship between K_m and K_d observed with *E. coli* photolyase and various homogeneous short-chain oligonucleotide substrates. Observed values for K_m/K_d (closed circles) are plotted versus K_d. The open squares are minimum values for K_m/K_d, calculated as described under Discussion.

In contrast to groups located immediately adjacent to the dimer, the presence of a 3′pT once removed from the dimer does not make any detectable contribution to the overall binding energy as evidenced by the similar binding energies observed with TpTp̂TpT and TpTp̂TpTpT. The binding energy observed for TpTpTp̂T is 0.3 kcal/mol greater than that observed for TpTp̂T, suggesting that the presence of a 5′Tp once removed from the dimer may make a small contribution to binding energy. On the basis of results obtained with isomers containing a single dimer, it was expected that the enzyme might show a preference for the dimer at the 5′ end in TpTp̂TpTp̂T. Consistent with this hypothesis, very similar binding energies are observed with TpTp̂TpTp̂T (7.0 kcal/mol) and TpTp̂TpTpT (6.9 kcal/mol). The results suggest that replacement of a pair of adjacent thymine residues with an additional dimer does not affect binding.

Steady-State Kinetic Studies with E. coli Photolyase. All of the dimer-containing oligothymidylate isomers discussed above function as substrates for photolyase. Our inability to detect dimer repair with pTp̂T in earlier studies (Jorns et al., 1985) was probably due to a combination of low substrate concentrations and shorter reaction times. Table I lists values obtained for K_m and k_{cat} at a saturating light intensity. The K_m values varied from 8.0 to 220 μM. The ratio K_m/K_d decreased from a maximum value of 22 with the most tightly bound isomer and appeared to plateau at a value near 1 with the weakest binding isomers (Figure 3). Despite large differences in binding energy, the various isomers exhibited similar values for k_{cat}. Turnover with these homogeneous short-chain oligonucleotide substrates (average k_{cat} = 6.7 min^{-1}) occurs at a rate similar to that observed with a mixture of isomers [UV-oligo$(dT)_{18}$] generated by UV irradiation of $(pT)_{18}$ (k_{cat} = 6.8 min^{-1}). Our previous studies have shown that the same turnover rate is observed with heterogeneous oligonucleotide substrates [UV-oligo$(dT)_n$, n = 4–18] and with UV-irradiated plasmid DNA (Jorns et al., 1985). The results suggest that there is a rate-determining step in catalysis which is unaffected by structural changes in the vicinity of the dimer which, nevertheless, can cause as much as a 25 000-fold variation in substrate binding constants.

Results obtained in earlier studies with a mixture of isomers generated by UV irradiation of pTpTpTpT (A, pTp̂TpTpT; B, pTpTp̂TpT; C, pTpTpTp̂T) led us to suspect that the en-

Table II: Reaction of Yeast Photolyase with Dimer-Containing Oligothymidylates

oligomer	substrate	K_m (μM)	k_{cat} (min^{-1})
T$\hat{p}$T	no[a]		
pT$\hat{p}$T	no[a]		
TpT$\hat{p}$T	poor		
TpTpT$\hat{p}$T	poor		
TpT$\hat{p}$TpT	yes	140	3.9
T$\hat{p}$TpT	yes	87	5.0
T$\hat{p}$Tp[b]	yes	72 (94)	4.8 (4.6)
T$\hat{p}$TpTpT	yes	5.2	5.2
T$\hat{p}$TpT$\hat{p}$T	yes	9.3	5.3
UV-oligo(dT)$_6$	yes	12	4.6
UV-oligo(dT)$_9$	yes	*c*	5.2
UV-oligo(dT)$_{18}$	yes	*c*	4.9
UV-DNA	yes		0.7[d]

[a] No activity was detected at the highest substrate concentration tested with T$\hat{p}$T (0.3 mM) or pT$\hat{p}$T (0.03 mM). [b] Values were obtained with substrate prepared by the method of Jorns et al. (1985). Results obtained with substrate prepared according to Rycyna et al. (1988) are shown in parentheses. [c] K_m values are too small to measure in the current assay. [d] Value reported by Sancar et al. (1987) as a minimal estimate since the enzyme was unstable under assay conditions.

zyme had a similar affinity for and could repair dimers in isomers A and B (K_d = 0.6 μM) but that the enzyme did not repair dimers in the less strongly bound isomer C (Jorns et al., 1985; Jordan & Jorns, 1988). Binding affinity predictions are consistent with results obtained with homogeneous substrates which allow us to estimate expected dissociation constants for the various isomers (A, 1 μM; B, 0.4 μM; C; 8 μM). On the other hand, our current studies indicate that the enzyme should be able to repair dimers in isomer C, although its K_m value would be significantly larger than those of the other two isomers. Taking into account an expected slower repair rate with isomer C under the conditions of the earlier experiment, we repeated the experiment using more enzyme and longer reaction times. Under these conditions we found that 95% of the dimers in UV-oligo(dT)$_4$ could be repaired, in contrast to earlier studies where repair of only two-thirds of the dimers was observed.

Steady-State Kinetic Studies with Yeast Photolyase. All homogeneous oligothymidylate isomers containing a phosphate immediately 3′ to the dimer function as substrates for yeast photolyase. Similar values are observed for k_{cat} with these isomers and with mixtures of longer chain isomers, such as UV-oligo(dT)$_n$ (n = 6, 9, 18) (Table II). Turnover with these oligonucleotide substrates (average k_{cat} = 4.8 min^{-1}) is faster than the rate observed with double-stranded plasmid DNA (0.7) (Sancar et al., 1987b), but the latter value was reported as a minimal estimate since measurements were made under conditions where the enzyme was unstable.

Yeast photolyase exhibits a near absolute requirement for phosphate immediately 3′ to the dimer. Isomers lacking a 3′p are either inactive or very poor substrates. For example, no dimer repair was observed with T$\hat{p}$T at the highest concentration tested (0.3 mM). The rate of dimer repair observed with 0.1 mM TpTpT$\hat{p}$T was only 2.7% of that observed with saturating T$\hat{p}$TpTpT (0.1 mM) or UV-oligo(dT)$_{18}$ (6.7 μM dimer). Not surprisingly, the enzyme repaired only half of the dimers in T$\hat{p}$TpT$\hat{p}$T. The K_m value observed with T$\hat{p}$TpT$\hat{p}$T was only 1.8-fold larger than the value observed with T$\hat{p}$TpTpT, suggesting that repair of the dimer at the 5′ end in T$\hat{p}$TpT$\hat{p}$T was not appreciably affected by the presence of an additional dimer at the 3′ end of the molecule.

Assuming that K_m values provide a measure of binding affinity, the similar values observed for K_m with TpT$\hat{p}$TpT or T$\hat{p}$TpT suggest that the presence of a phosphate or a thymine residue immediately 5′ to the dimer has little effect on binding to yeast photolyase. On the other hand, the 17-fold difference in K_m values observed with T$\hat{p}$TpT versus T$\hat{p}$TpTpT suggests that the presence of a pT moiety once removed from the 3′ end of the dimer does contribute to binding affinity. The K_m observed for UV-oligo(dT)$_6$ (0.9 mol of dimer/mol of oligomer) represents an average value for five different isomers, including at least one isomer (pTpTpTpTpT$\hat{p}$T) that is expected to be a very poor substrate for the enzyme. In the case of UV-oligo(dT)$_n$ (n = 9, 18), K_m values were too small to measure with our current assay.

Discussion

The stability of the complex formed with T$\hat{p}$T and *E. coli* photolyase (ΔG = 4.6 kcal/mol) suggests that about 45% of the binding energy observed with the enzyme and UV-irradiated plasmid DNA [$\Delta G \sim$ 10 kcal/mol (Sancar et al., 1987c)] can be attributed to interaction with the dimer itself. This is compatible with other studies which show that *E. coli* photolyase recognizes the dimer rather than the deformation in DNA structure produced by the dimer (Jorns et al., 1985). Additional interaction of the enzyme with phosphate and thymine residues immediately 5′ and 3′ to the dimer accounts for the increased binding energy observed with TpT$\hat{p}$TpT as compared with T$\hat{p}$T. The observed binding energy with TpT$\hat{p}$TpT (ΔG = 8.2 kcal/mol) suggests that about 80% of the binding energy observed with DNA as substrate can be attributed to the interaction of the enzyme with a dimer-containing region that spans only four nucleotides in length. This major binding determinant coincides with the major conformational impact region of the dimer as judged by NMR studies which show that the perturbation caused by the introduction of a dimer into a single-stranded oligonucleotide is limited to the dimer itself plus nucleotides immediately adjacent to the dimer but has little effect on nucleotides further removed (Rycyna et al., 1988). Similar results are obtained with duplex oligonucleotides which show that the dimer also perturbs a comparable region in the complimentary strand (Kemmink et al., 1987). The difference in binding energy ($\sim$2 kcal/mol) observed for TpT$\hat{p}$TpT versus dimer-containing DNA or higher oligothymidylates [UV-oligo(dT)$_n$, $n \geq 9$] may reflect multiple weak interactions with groups somewhat distant from the dimer. Our current studies suggest that there may be a weak interaction (0.3 kcal/mol) with a Tp unit once removed from the dimer on the 5′ side.

Our evidence for electrostatic interaction of *E. coli* photolyase with phosphates immediately 3′ and 5′ to the dimer in oligonucleotide substrates is fully compatible with data obtained in footprint analysis studies with dimer-containing DNA (Husain et al., 1987). On the other hand, footprint analysis studies suggest that the major contact between the enzyme and UV-damaged DNA (NpT$\hat{p}$TpNpNpN) is two nucleotides longer on the 3′ side of the dimer as compared with the major binding determinant (NpT$\hat{p}$TpN) estimated with oligonucleotide substrates. Although we find that interaction energetics are somewhat more favorable for a phosphate and a thymine residue located immediately 3′ to the dimer as compared with equivalent groups on the 5′ side, we could not detect any contribution from a phosphate or a thymine residue once removed from the dimer on the 3′ side.

Studies with *E. coli* photolyase show that k_{cat} is virtually independent of substrate binding affinity. On the other hand, the ratio K_m/K_d decreases as binding affinity decreases and appears to plateau at a value near 1 with weakly bound substrates. A minimal reaction mechanism is suggested in eq 1 which makes the reasonable assumption that the photochem-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[\text{light}]{k_2} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \quad (1)$$

ical step in catalysis is irreversible. The rate of the photochemical step can be estimated since $k_2 = k_pI$ (k_p = photolytic constant, I = light intensity), and the photolytic constant was determined in our previous studies using plasmid DNA as substrate (Sancar et al., 1987a). The value calculated for k_2 (5.1 min^{-1}) at saturating light is similar to the turnover rate observed with DNA and with various oligonucleotide substrates, suggesting that $k_{cat} = k_2$. This relationship is compatible with the mechanism shown in eq 1 [$k_{cat} = k_2k_3/(k_2 + k_3)$], provided that $k_2 \ll k_3$. The expression for K_m [$K_m = k_3(k_{-1} + k_2)/k_1(k_2 + k_3)$] is simplified [$K_m = (k_{-1} + k_2)/k_1$] when $k_2 \ll k_3$. Under these conditions, K_m/K_d will approach unity when $k_{-1} > k_2$ (eq 2). A minimum value for k_{-1} can

$$K_m/K_d = 1 + k_2/k_{-1} \quad (2)$$

be determined since K_d values are known for each of the ES complexes, and a minimum value for k_1 can be estimated from the ratio k_{cat}/K_m, which, in general, sets a lower limit for any second-order rate constant on the forward reaction pathway (Peller & Alberty, 1959). The estimated minimum values for k_{-1} increase as substrate binding affinity decreases and approach the observed value for k_{cat} in the case of the weakly bound substrates (Table I). Minimum values for the ratio K_m/K_d were then estimated from the minimum values for k_{-1} and eq 2 [$k_2 = k_{cat}$ (6.7 min^{-1})]. The calculated values are in moderately good agreement with the observed data (Figure 3), suggesting that the actual values for k_{-1} may be close to our minimal estimates. In the case of the tighter binding substrates, actual values for k_1 and k_{-1} can be estimated from observed values for K_d, K_m, and eq 2. In each case, the calculated values are close to the estimated minimum values for these rate constants (Table I). This analysis suggests that a decrease in binding energy with oligonucleotide substrates is due to the combined effects of slower binding and faster dissociation of the ES complex. If a similar relationship applies for dimer-free products, the dissociation of the EP complex (k_3) should be very fast since the binding of unmodified oligonucleotides is extremely weak and not readily detectable (Jordan & Jorns, 1988). A fast dissociation of product would be compatible with our other data, which suggest that the photochemical step in catalysis is rate determining.

Although less extensive studies were conducted with yeast photolyase, the results reveal certain intriguing similarities as well as differences with the *E. coli* enzyme. Both enzymes can repair dimers in oligomers containing as few as two thymine residues. In the case of yeast photolyase, this finding does not support a prediction made by Setlow and Bollum (1968) that the enzyme would not repair dimers in short or medium-length oligomers [UV-oligo(dT)$_n$ ($n < 9$)]. Despite significant differences in K_m values, similar turnover rates are observed with yeast (k_{cat} = 4.8 min^{-1}) or *E. coli* (k_{cat} = 6.7 min^{-1}) photolyase and various dimer-containing oligothymidylates that function as substrates for both enzymes. The latter include all isomers tested except those lacking a phosphate immediately 3′ to the dimer which are poor substrates for yeast photolyase. Although phosphate and thymine residues immediately adjacent to the dimer contribute significantly to binding affinity with *E. coli* photolyase, none of these residues is absolutely essential for binding. In contrast, the yeast enzyme exhibits a near absolute requirement for phosphate immediately 3′ to the dimer whereas phosphate or thymine immediately 5′ to the dimer does not appear to contribute to binding affinity. A further difference is seen with phosphate and thymine residues once removed from the 3′ end of the dimer which are important for binding with yeast photolyase but not with the *E. coli* enzyme. The results obtained with yeast photolyase suggest that the major binding determinant (TpTpTpT) is different from that observed for *E. coli* photolyase (TpTpTpT). Additional studies are needed to determine whether the observed differences in substrate specificity might be related to an adaptation of the eukaryotic yeast enzyme for dimer repair with chromosomal DNA as substrate. In this connection, it may be relevant that yeast photolyase contains a 73 amino acid tail at the N-terminal end that is absent in prokaryotic photolyases, although a high degree of amino acid sequence homology is otherwise observed for eukaryotic and prokaryotic enzymes (Yasui et al., 1988).

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