Identification of Oligothymidylates as New Simple Substrates for *Escherichia coli* DNA Photolyase and Their Use in a Rapid Spectrophotometric Enzyme Assay[†]

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Received September 17, 1984

ABSTRACT: *Escherichia coli* DNA photolyase exhibits the same turnover number (3.4 min^{-1}) for the repair of dimers in oligothymidylates [oligo$(\mathrm{dT})_n$] containing 4–18 thymine residues. This rate is identical with that observed with polythymidylate and with native DNA. The enzyme exhibits a similar high affinity with oligomers containing seven or more thymine residues. A decrease in affinity is detectable with oligo$(\mathrm{dT})_n$ when $n = 4$–6. The enzyme is active with oligo$(\mathrm{dT})_3$, but no evidence for saturation was obtained at dimer concentrations up to 15 μM where the observed repair rate is 43% of the turnover number observed with the higher homologues. Nearly quantitative (90–100%) repair is observed with oligo$(\mathrm{dT})_n$ when $n \geq 9$. Photolyase can repair internal dimers and dimers at a 5′ end where the terminal ribose is phosphorylated but not at unphosphorylated 5′ or 3′ ends. The latter can explain a progressive decrease in the extent of repair observed with short-chain oligomers. The observed specificity can also explain why the enzyme is inactive with oligo$(\mathrm{dT})_2$ [p$(\mathrm{dT})_2$] since the only dimer possible in oligo$(\mathrm{dT})_2$ involves an unphosphorylated 3′ end. That the enzyme can repair dimers in short-chain, single-stranded analogues for DNA suggests that in catalysis with DNA recognition of the dimer itself is important as opposed to recognition of the deformation in DNA structure produced by the dimer. Dimer repair with oligo$(\mathrm{dT})_n$ is detected by the increase in absorbance at 260 nm, a feature which is used as the basis for a rapid spectrophotometric assay with a lower detection limit around 150 pmol of dimer repaired.

Exposure of DNA to ultraviolet light results in the formation of dimers between adjacent pyrimidine bases in a photochemically reversible reaction. The dimers can be monomerized with shorter wavelengths of ultraviolet light or by DNA photolyase (E) in an enzymic reaction (eq 1) which

$$\mathrm{E} + \mathrm{DNA\ (UV)} \underset{k_2,\ \mathrm{dark}}{\overset{k_1,\ \mathrm{dark}}{\rightleftharpoons}} \mathrm{E{\cdot}DNA\ (UV)} \xrightarrow[\mathrm{light}]{k_3} \mathrm{E} + \mathrm{DNA} \qquad (1)$$

requires visible light. Since direct excitation of the dimers does not occur with visible light, it has been proposed that the photolyase reaction is a photosensitized process involving an enzyme–bound chromophore which absorbs in the visible region. This hypothesis has been difficult to evaluate since the enzyme is typically present at very low levels in most tissues. A major breakthrough in the study of DNA photolyase was provided by cloning techniques which have generated a strain of *Escherichia coli* where photolyase constitutes 15% of the total protein (Sancar et al., 1984a). Recent studies by Jorns et al. (1984) have shown that the *E. coli* enzyme contains two chromophores, a blue flavin adenine dinucleotide (FAD)[1] radical and a partially characterized second chromophore which is probably responsible for the fluorescence of the enzyme. In order to facilitate studies on the nature of the interaction of the enzyme with DNA and on the role of the chromophores in catalysis, we initiated a search for simple DNA analogues which might function as substrates for photolyase. It was hoped that such studies might also lead to the development of a new assay for the enzyme. Existing assays (Sancar et al., 1984a; Rupert et al., 1958; Cook & Worthy, 1972; Sutherland & Chamberlin, 1972; Farland & Sutherland, 1979; Madden et al., 1973) generally use bacterial, phage, or plasmid DNA as substrate and are quite sensitive but, unfortunately, are relatively slow and/or require time-consuming preparation of radioactive substrates. Assays able to detect as little as 1 pmol of dimer repaired were developed for the isolation of photolyase from cells containing very small amounts of enzyme. The availability of relatively large amounts of the *E. coli* enzyme could justify an alternative assay where sensitivity might be partially sacrificed in favor of speed and, ideally, commercially available substrates. Rupert & To (1976) obtained indirect evidence to show that thymine dimers in poly(dA)·poly(dT) could be monomerized by yeast photolyase, indicating that the enzyme was active with substrates other than natural DNA. In 1968, Setlow & Bollum (1968) showed that UV-irradiated oligothymidylates [oligo$(\mathrm{dT})_n$, $n \geq 9$] were bound to yeast photolyase as evidenced by competition data obtained by using a transformation assay with *Hemophilius influenzae* DNA as substrate. In this paper, we show that dimers in oligo$(\mathrm{dT})_n$ ($n \geq 3$) are mo-

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[†] This work was supported in part by Grants GM31704 (M.S.J.) and GM31082 (A.S.) from the National Institutes of Health.

[1] Abbreviations: oligo$(\mathrm{dT})_n$, oligothymidylate; poly(dT), polythymidylate; FAD, flavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

0006-2960/85/0424-1856$01.50/0

nomerized by *E. coli* DNA photolyase and describe a rapid spectrophotometric assay using these compounds as substrate.

EXPERIMENTAL PROCEDURES

Materials. Oligothymidylates [oligo(dT)$_n$, p(dT)$_n$, n = 2–7, 9, 14, and 18], polythymidylate [poly(dT), $s_{20,w}$ = 7.55 S], and dephosphooligo(dT)$_4$ (no 5′-phosphate) were obtained from P-L Biochemicals.

Methods. Cloned *E. coli* DNA photolyase was isolated by a procedure described elsewhere (Sancar et al., 1984a). Concentrated stock solutions of enzyme were stored at −80 or −20 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol. Except where noted, all handling of the enzyme was done under yellow light.

Stock solutions of oligo(dT)$_{18}$ (5.9×10^{-5} M) in 10 mM Tris-HCl, pH 7.2, containing 0.5 mM EDTA were irradiated in stoppered quartz semimicro cuvettes (250 µL/cuvette). The cuvettes were laid horizontally on an optical face and irradiated from above for 6 min at 4 °C with shaking (Tektator V shaker) at a distance of 5 cm from two germicidal lights (Sylvania G15T8, 15 W). Dimer formation was monitored by decreases in absorbance at 290 or 295 nm for concentrated solutions of oligo(dT)$_n$ or at 260 nm for dilute solutions. The same procedure was used for irradiation with other oligothymidylates (n = 2–7, 9, and 14) and with polythymidylate. These experiments were conducted at a constant thymine residue concentration (1.06×10^{-3} M) equivalent to that obtained with 5.9×10^{-5} M oligo(dT)$_{18}$.

Except where noted, enzyme assays with oligo(dT)$_{18}$ (2.34×10^{-6} M, containing 34–37% dimer) were conducted by using a 250-µL reaction volume under conditions (50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl and 1 mM EDTA, 20 ± 1 °C) similar to those used in the transformation assay described by Sancar et al. (1984a) except that dithiothreitol was omitted because it interferred with optical measurements at 260 nm. Solutions containing enzyme plus cold assay mixture were placed in a stoppered semimicro cuvette, incubated in the dark for 3 min at 20 ± 1 °C, and then illuminated with the cuvettes in a horizontal position at a distance of 5 cm from two black lights (Sylvania F15T8/BLB, 15 W). The same conditions were used for reactions with other oligothymidylates and with polythymidylate. The dimer content in these substrates will be discussed under Results. Except where noted, the experiments were conducted at a constant thymine residue concentration (4.21×10^{-5} M) equal to that obtained with 2.34×10^{-6} M oligo(dT)$_{18}$. Enzyme activity (picomoles of dimer repaired) was calculated from the change in absorbance at 260 nm by using an extinction coefficient ($\epsilon_{260} = 8.3 \times 10^3$ M^{-1} cm^{-1}) obtained by correcting a value reported (Naylor & Gilham, 1966) for oligo(dT)$_{10}$ at 266 nm for the 7% lower absorbance observed with oligo(dT)$_n$ at 260 nm. The same value was used for all compounds tested since the value observed with oligo(dT)$_{10}$ is only slightly affected by either decreasing [3% lower with oligo(dT)$_2$ (Naylor & Gilham, 1966)] or increasing [3% higher with poly(dT) (Ts'o et al., 1966)] the chain length.

In calculating the dimer distribution shown in Table I, it was assumed that all pairs of adjacent thymidine residues are equally good targets for dimer formation. For oligo(dT)$_n$, there are $n - 1$ types of molecules containing one dimer. The probability of having any one of these types is $[1/(n-1)]P_1$ where P_1 is the sum of the probabilities for all types in this class. With long polynucleotide chains, the probability of having two dimers per molecule, P_2, is equal to $P_1{}^2$. This is not the case with short oligonucleotides where the first dimerization event results in a substantial reduction in the number of target sites in a manner which will depend on the position of the first dimer in the molecule. The probability of introducing a second dimer into a short oligonucleotide containing one dimer at a specific location in the molecule can be calculated by using the following equation: $P_{2j} = [1/(n-1)]P_1xP_1$ where x is the fraction of remaining dimer sites as compared to those available for the first dimerization event. The process is repeated for all types of molecules containing one dimer, and P_2 is calculated by summing all values for P_{2j}. In a similar fashion, the probability of introducing a third dimer, P_3, can be determined in terms of P_1 by considering all types of molecules containing two dimers. Absolute values for P_1, P_2, and P_3 can be calculated by using the interrelationships determined as described above and the equation $A = \sum_0^m d(P_d)$ where d is the number of dimers per molecule, P_d is the probability of having d dimers per molecule, m is the maximum possible number of dimers per molecule, and A is the average number of dimers per molecule. A can be calculated from the fraction of total thymidines as dimers (F) by using the relationship $A = Fn/2$ where n is the number of thymidine residues per molecule. The proportion of dimers located at a 5′ end is determined by summing the probabilities for all molecules containing a dimer at the 5′ end and then dividing this sum by A, the average number of dimers per molecule. The proportion of dimers at the 3′ end and at internal positions is determined in a similar fashion.

Table I: Extent of Dimer Repair with Short-Chain Oligothymidylates[a]

	expected dimer distribution (%)				
n[b]	internal	5′ end	3′ end	internal + 5′ end	extent of enzymic repair (%)
7	63 (67)	19 (17)	19 (17)	82 (83)	78
6	56 (60)	22 (20)	22 (20)	78 (80)	81
5	46 (50)	27 (25)	27 (25)	73 (75)	71
4	28 (33)	36 (33)	36 (33)	64 (67)	54
3	0 (0)	50 (50)	50 (50)	50 (50)	36

[a] The expected dimer distribution is calculated (see Methods for details) by assuming that all pairs of adjacent residues are equally good targets for dimer formation. Values in parentheses are calculated for the case of molecules containing one dimer. [b] n refers to the number of thymine residues per molecule of oligo(dT)$_n$.

RESULTS

Oligo(dT)$_{18}$ was selected for initial studies since the data of Setlow & Bollum (1968) on the yeast enzyme showed that this was the shortest chain oligomer which exhibited binding properties similar to that observed with poly(dT). In comparison with thymine, thymine dimers do not exhibit significant absorption at 260 nm. Irradiation of oligo(dT)$_{18}$ with light at 254 nm results in the rapid formation of thymine dimers as evidenced by decreases in absorption at 260 nm. At a maximum, 37% conversion to dimers was observed which compares favorably with results obtained by Deering & Setlow (1963) with poly(dT) (39% dimer) and oligo(dT)$_2$ (33% dimer). In contrast, a maximum of 7% dimers is formed with DNA under similar conditions (Patrick & Rahn, 1976). If the oligomer functioned as a substrate for photolyase, the high dimer content and the absence of interference due to purine absorption found in DNA or poly(dT)·poly(dA) could be useful properties in developing a rapid spectrophotometric assay for the enzyme. Indeed, it was found that illumination of UV-irradiated poly(dT)$_{18}$ with black light in the presence of *E. coli* DNA photolyase results in an increase in the absorption at 260 nm to a level identical with that observed before UV irradiation. Studies with varying amounts of photolyase

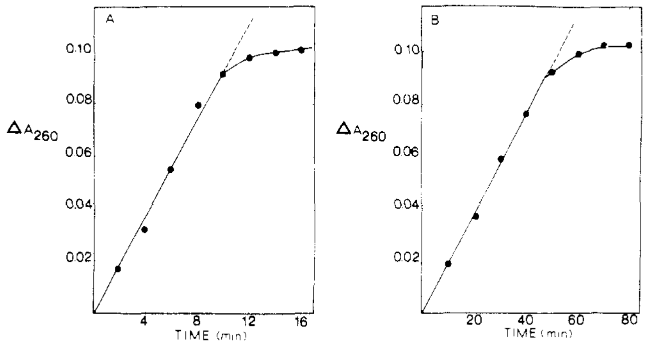

FIGURE 1: Effect of illumination time on the extent of dimer repair observed with oligo(dT)$_{18}$. Results obtained with 1.7 × 10^{-7} and 3.3 × 10^{-8} M photolyase are shown in panels A and B, respectively.

(3.3 × 10^{-8} to 3.3 × 10^{-7} M) show that the rate of dimer monomerization, as measured by the increase in absorbance at 260 nm observed after a 5-min illumination with black light, is directly proportional to enzyme concentration. Control studies show that no increase in absorption at 260 nm occurs when UV-irradiated oligo(dT)$_{18}$ is illuminated with black light in the absence of photolyase or with enzyme previously denatured by boiling for 5 min. No changes at 260 nm are observed upon incubation of native enzyme with UV-irradiated oligo(dT)$_{18}$ in the dark or upon illumination of solutions containing native enzyme and unirradiated oligo(dT)$_{18}$. The results show that black light and native photolyase are required for monomerization of dimers in oligo(dT)$_{18}$.

Figure 1A shows the time course of dimer repair observed with 1.7 × 10^{-7} M photolyase and 2.38 × 10^{-6} M oligo(dT)$_{18}$ containing 7.86 × 10^{-6} M dimers (34% dimer). A linear rate is observed for the first 10 min which corresponds to 90% of the reaction. The rate decreases as dimer repair nears completion. The reaction is catalytic as evidenced by the fact that the enzyme is able to repair dimers present in 40-fold excess. That the decrease in rate observed during the last 10% of the reaction is due to substrate depletion and not enzyme inactivation is consistent with results obtained when the experiment is repeated using one-fifth the amount of enzyme (Figure 1B). In this case, the rate is linear for 50 min and then curves off during the last 10% of the reaction. The results in Figure 1 indicate that the initial substrate concentration is saturating, a conclusion which is supported by additional experiments which show that the reaction rate is unaffected by doubling or tripling the substrate concentration (data not shown). The data were used to calculate a turnover number of 3.4 min^{-1} when enzyme concentration is based on its flavin content (Jorns et al., 1984) or 2.4 min^{-1} when calculated on the basis of protein since the preparation contains 0.7 mol of flavin/mol of protein (Jorns et al., 1984). The latter calculation is comparable to that used in previous studies where a turnover number of 2.4 min^{-1} was reported (Sancar et al., 1984a) for the cloned *E. coli* enzyme on the basis of a transformation assay with plasmid DNA as substrate under reaction conditions otherwise similar to the spectrophotometric assay. The results indicate that oligo(dT)$_{18}$ is remarkably comparable to natural DNA as a substrate for the enzyme. This means that turnover is not significantly affected by the fact that DNA is double stranded. That the reaction rate observed with oligo(dT)$_{18}$ is constant until the dimer concentration falls to about 8 × 10^{-7} M suggests that the K_m for oligo(dT)$_{18}$ is very small. A K_m value for the *E. coli* enzyme with DNA as substrate has not been reported, but a value around 10^{-8}–10^{-9} M can be estimated for the dissociation constant of the enzyme–DNA complex on the basis of values for k_1 and k_2 (see eq 1) obtained in studies with *E. coli* cells (Harm & Rupert, 1968).

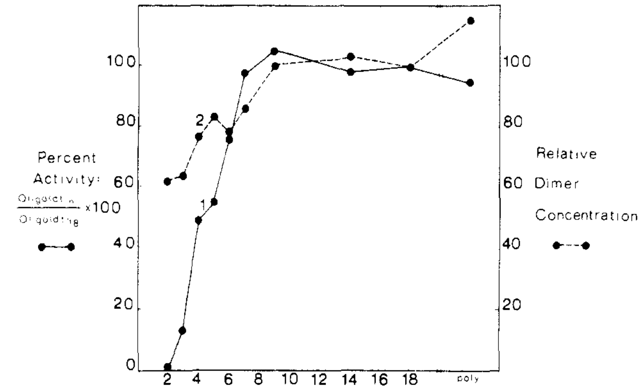

FIGURE 2: Activity of photolyase with various oligothymidylates and polythymidylates. Activity, expressed as a percent of the rate observed with oligo(dT)$_{18}$, is plotted (curve 1) vs. *n*, the number of thymine residues per molecule of oligo(dT)$_n$ or poly(dT). All reactions were conducted at the same initial thymine residue concentration (4.21 × 10^{-5} M). Dimer content relative to that observed with oligo(dT)$_{18}$ (37%) is plotted vs. *n* in curve 2.

With double-stranded DNA, the introduction of a dimer results in denaturation of about 4 base pairs (Kelley et al., 1969; Hayes et al., 1971). The ability of photolyase to seek out and repair dimers in DNA might reflect the ability of the enzyme to recognize the particular deformation in DNA structure caused by the dimer. Alternatively, instead of the distortion in the DNA, the critical factor may be recognition of the dimer itself. The latter hypothesis is supported by the fact that the enzyme exhibits similar activity with single-stranded oligo(dT)$_{18}$ and duplex DNA. Further evidence was sought by examining the effect of chain length on activity. If dimer recognition is a critical factor, the enzyme might also be expected to repair dimers in short-chain oligothymidylates. An initial survey was made in which all homologues were tested at the same thymine residue concentration (4.21 × 10^{-5} M) used in the studies described above with oligo(dT)$_{18}$. Controls, performed with oligo(dT)$_{18}$, were repeated with selected homologues [poly(dT) and n = 3, 5, 9, and 14 with oligo(dT)$_n$] to show that the observed reactions required both light and native photolyase. As shown in Figure 2, increasing the chain length in going from oligo(dT)$_{18}$ to poly(dT) does not affect activity. This result was not unexpected since the enzyme exhibits similar activity with oligo(dT)$_{18}$ and DNA. As observed with oligo(dT)$_{18}$, assays with poly(dT) are linear for at least 90% of the reaction, and activity is unaffected by doubling or tripling the substrate concentration (data not shown). Similar results are obtained with oligo(dT)$_{14}$, oligo(dT)$_9$, and oligo(dT)$_7$ which appear to be as good as substrates as the higher homologues. Starting with oligo(dT)$_6$, a progressive decrease in activity is observed in going to derivatives containing five, four, and three thymine residues, respectively, per molecule. No activity was detected with the lowest member of the series, oligo(dT)$_2$. The substrates used in these studies were prepared by irradiating with light at 254 nm under conditions which produced a maximum amount of dimers with oligo(dT)$_{18}$. Under these conditions, a similar yield of dimers was observed with oligo(dT)$_n$ at $n \geq 9$ [oligo(dT)$_{18}$, 37%; oligo(dT)$_{14}$, 38%; oligo(dT)$_9$, 37%]. However, a progressive decrease in dimer content was observed with the lower homologues [oligo(dT)$_7$, 32%; oligo(dT)$_6$, 29%; oligo(dT)$_5$, 31%; oligo(dT)$_4$, 28%; oligo(dT)$_3$, 24%; oligo(dT)$_2$, 23%]. As a consequence, although the data in Figure 2 were obtained at a constant thymine residue concentration, the reactions with the lower homologues were actually conducted

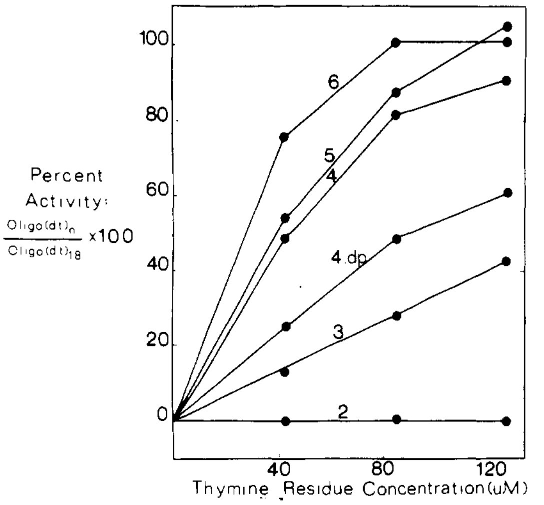

FIGURE 3: Effect of substrate concentration on the activity observed with short-chain oligothymidylates. Activity is expressed as a percent of the rate observed with oligo(dT)$_{18}$. The latter was measured under the same conditions as described in the legend to Figure 2. Data obtained with oligo(dT)$_{2-6}$ are shown in curves 2–6, respectively. Results with dephosphooligo(dT)$_4$ are shown in curve 4, dp.

at a progressively lower dimer concentration. Although the decrease was relatively modest [at a maximum, 38% lower with oligo(dT)$_2$ as compared to oligo(dT)$_{18}$], it might contribute to the lower activity observed with some of the shorter chain derivatives if the substrates were present at less than saturating concentrations. That this was probably a major factor in the case of oligo(dT)$_6$ is evidenced by the fact that doubling the substrate concentration results in a rate identical with that observed with oligo(dT)$_{18}$ (Figure 3). While more detailed studies with the higher homologues might reveal differences in K_m values, within the limits of resolution of the present studies, the data with oligo(dT)$_6$ provide the first indication for an increase in K_m as the series is descended. A larger K_m is clearly evident in the case of oligo(dT)$_5$ and oligo(dT)$_4$ where reaction rates appear to approach that observed with oligo(dT)$_{18}$ but only at substrate concentrations at least 3 times the level used in the initial survey. Within the substrate range tested, no evidence for saturation was observed with oligo(dT)$_3$, where tripling the substrate concentration results in an increase in activity from 13% to 43% of the rate observed with oligo(dT)$_{18}$. With oligo(dT)$_2$, no activity was detected even at higher substrate concentrations.

That the *E. coli* enzyme binds and efficiently repairs dimers in an oligonucleotide as short as oligo(dT)$_4$ provides strong evidence that a key feature in catalysis involves recognition of the dimer rather than the distortion produced by the dimer in the DNA structure. In this case, it is puzzling that the enzyme is unable to repair dimers in oligo(dT)$_2$. This result appears to be related to the effect of chain length on the extent of dimer repair. Nearly quantitative repair is observed with oligomers containing nine or more thymine residues [oligo(dT)$_9$, 91%; oligo(dT)$_{14}$, 104%; oligo(dT)$_{18}$, 101%; poly(dT), 90%]. With the lower homologues, a progressive decrease in the extent of repair is observed, reaching a low value at 36% repair with oligo(dT)$_3$ (Table I). Unlike native DNA, where only cis-syn pyrimidine dimers are formed, it has been reported (Rahn & Landry, 1971; Ben-Hur & Ben-Ishai, 1968) that approximately 10% of the dimers formed with denatured DNA or with poly(dT) are present as the trans-syn isomer which cannot be monomerized by the photolyase from yeast (Ben-Hur & Ben-Ishai, 1968). This factor clearly cannot account for the results obtained with the *E. coli* enzyme and the short-chain oligomers. On the other hand, it is useful to consider that certain structural properties of oligothymidylates, which detract from the usefulness of these compounds as models for a short segment of a polymer, can become significant in the case of the shorter chain homologues. In particular, with the lower homologues internal thymine residues constitute a progressively smaller portion of the molecule as compared with residues at the 5′ and 3′ ends. Thus, with oligo(dT)$_7$, nearly 70% of the residues are internal whereas with oligo(dT)$_2$ there are no internal residues. It is readily apparent that internal residues and those located at the 5′ and 3′ ends are structurally nonequivalent. The decrease in the extent of dimer repair observed with the shorter chain homologues might reflect the inability of the enzyme to repair a dimer located at the 5′ and/or 3′ end. Table I compares the observed extent of dimer repair with the expected distribution of dimers at internal positions and at the 3′ and 5′ ends. The expected distribution was calculated (see Methods) by assuming that all pairs of adjacent residues are equally good targets for dimer formation. The extent of dimer repair observed with photolyase and oligomers containing four to seven thymine residues exceeds the maximum percent of dimers involving only internal thymine residues, indicating that the enzyme must be capable of repairing at least some dimers which involve a terminal residue. The latter is also consistent with the fact that the enzyme is active with oligo(dT)$_3$, a homologue where internal dimers are not possible. The observed extent of repair agrees reasonably well with that expected for repair of internal dimers plus selective repair of dimers involving a residue at only one of the two terminal positions. In the case of the oligomers used in these studies [p(dT)$_n$], a thymine residue at the 5′ end is attached to a deoxyribosyl moiety containing phosphate groups at both the 3′- and 5′-positions and, in this sense, more closely resembles an internal residue as compared to a thymine at the opposite end where the corresponding deoxyribosyl moiety contains an unsubstituted 3′-hydroxyl group. This difference might provide the basis for selective repair of dimers at the 5′ end. To test this hypothesis, studies were conducted with dephosphooligo(dT)$_4$ [(dT)$_n$] where the deoxyribosyl moiety at the 5′ end contains an unsubstituted 5′-hydroxyl group. [When irradiated with ultraviolet light, under the same conditions used for other oligomers, the dimer yield with dephosphooligo(dT)$_4$ (28%) was identical with that observed with oligo(dT)$_4$.] As compared with oligo(dT)$_4$ (54 ± 2.8% repair), a considerable decrease in the extent of dimer repair is observed with dephosphooligo(dT)$_4$ (35 ± 2.9% repair). The latter value agrees reasonably well with the expected content of internal dimers (28%). The small discrepancy between observed and predicted values might be related to the assumption used in calculating the expected dimer distribution. If pairs of adjacent residues are much poorer targets for dimer formation when present in a molecule already containing one dimer, it might be more valid to calculate a distribution by assuming that no molecules contain more than one dimer. The latter condition is reasonable in terms of the number of residues per molecule and the observed percent of residues present as dimers. While excluding molecules containing more than one dimer causes only a small change in the expected distribution (see Table I), it does increase the expected content of internal dimers in dephosphooligo(dT)$_4$ (from 28% to 33%) to a value which agrees quite closely with the observed extent of repair. The results suggest that the enzyme is able to repair dimers at the 5′ end provided that the 5′-hydroxyl group is phosphorylated and does not repair dimers at the 3′ end when the 3′-hydroxyl group is unsubstituted. These results can explain the inability of the enzyme to repair dimers in oligo(dT)$_2$ since the only dimer possible in oligo(dT)$_2$ involves a 3′ end with an unsubstituted 3′-hydroxyl group.

DISCUSSION

The repair of dimers in polythymidylate and various oligothymidylates, observed with *E. coli* photolyase, is readily monitored by the increase in absorbance at 260 nm, a feature which has provided the basis for a rapid spectrophotometric assay. Under the reaction conditions described in studies with oligo(dT)$_{18}$ as substrate, absorbance changes at 260 nm in the range from 0.01 to 0.10 are easily measured. With a 250-μL assay, this corresponds to 150–1500 pmol of dimer repaired. With 8.3 pmol of enzyme, the lower detection limit is reached in about 5 min (Figure 1B). If necessary, even smaller amounts of enzyme could be assayed by increasing the reaction time since the enzyme is stable for at least 50 min under assay conditions. While less sensitive than other photolyase assays, the oligo(dT)$_{18}$ assay requires only modest amounts of enzyme and offers the advantage of speed and convenience since the substrate is commercially available. Although originally developed with oligo(dT)$_{18}$, the assay procedure is equally suitable and more economical when used with various shorter chain oligomers which exhibit properties similar to oligo(dT)$_{18}$ as substrates for photolyase.

The same turnover number is observed with photolyase and oligothymidylates containing 4–18 thymine residues per molecule. The rate with these substrates is identical with that observed with polythymidylate or with native DNA. That the enzyme is active with single-stranded substrate analogues is consistent with earlier in vivo studies with *E. coli* involving photorepair of UV-irradiated single-stranded ϕX174 DNA (Mennigmann, 1975). Repair of dimers in denatured DNA has been detected in in vitro studies with yeast photolyase where the rate observed was 25% of the rate with native DNA (Setlow, 1964). The high affinity observed for the *E. coli* enzyme with oligo(dT)$_{18}$ as substrate is similar to that observed by Setlow & Bollum (1968) with the yeast enzyme where it was found that oligo(dT)$_{18}$ was actually somewhat more effective than calf thymus DNA in competition studies with *Hemophilus influenzae* DNA as substrate. Although differences might be detected in more detailed studies, high affinity, similar to that observed with oligo(dT)$_{18}$, was observed for the *E. coli* enzyme with poly(dT) and oligomers containing seven or more thymine residues. With the yeast enzyme, a progressive decrease in binding affinity was observed with oligo(dT)$_{14}$ and oligo(dT)$_9$, and no binding was detected with oligo(dT)$_5$. The first evidence for a decrease in binding affinity with chain length was obtained for the *E. coli* enzyme with oligo(dT)$_6$ as substrate. With this enzyme, an apparently large decrease in affinity occurs in going from oligo(dT)$_4$ to oligo(dT)$_3$. While the basis for this observation is not immediately apparent, it may be related to the fact that oligo(dT)$_3$ is the only compound in the series which functions as a substrate but which cannot contain an internal dimer. It is conceivable that the enzyme might exhibit a much higher K_m for repair of dimers at a phosphorylated 5′ end as compared with internal dimers. In this case, initial rate measurements with a substrate such as oligo(dT)$_4$, which contains a mixture of internal and 5′-terminal dimers, would reflect kinetic parameters associated with repair of internal dimers whereas with oligo(dT)$_3$ the data could only be due to repair of dimers at the 5′ end. In this context, it should be noted that the K_m value associated with repair of internal dimers may be affected by the nature of nearby substituents. In particular, although further studies are needed, the data with dephosphooligo(dT)$_4$ tentatively suggest that removal of the phosphate from the 5′ end of oligo(dT)$_4$ results in a significant increase in the K_m value (Figure 3).

Studies on the extent of dimer repair as a function of chain length and on the effect of removing phosphate from the 5′ end suggest that photolyase is able to repair internal dimers and dimers located at a phosphorylated 5′ end but not those situated at unphosphorylated 5′ or 3′ ends. The latter can explain why oligo(dT)$_2$ is not a substrate for the enzyme. The data with all other nucleotides tested indicate that the enzyme is able to efficiently repair dimers in compounds which correspond to short-chain, single-stranded analogues for DNA. This strongly suggests that recognition of the dimer itself is a critical factor in catalysis with native DNA as opposed to recognition of the deformation in the DNA structure caused by the dimer. This in turn suggests that the photolyase binding site might involve a limited region immediately surrounding the dimer. This is apparently not the case with the UvrABC excision nuclease, an enzyme which removes dimers from DNA by cutting the eighth phosphodiester bond at the 5′ end of the dimer and the fourth or fifth phosphodiester bond at the 3′ end of the dimer. [The gap, created by the release of the dimer-containing oligonucleotide, is subsequently closed by DNA polymerase and ligase (Sancar & Rupp, 1983).] The cutting sites of the excision nuclease are rotated with respect to the dimer and are not on the same face of the DNA molecule, suggesting that the enzyme might not recognize the dimer but rather the DNA distortion. This view is supported by the fact that the nuclease acts on DNA adducts caused by a wide variety of agents (e.g., psoralen, *cis*-dichlorodiammineplatinum, nitrous acid, mitomycin C) that have no resemblance to pyrimidine dimers. In addition, the nuclease requires double-stranded DNA and will not accept single-stranded DNA as substrate (Sancar et al., 1984b). The hypothesis that photolyase and UvrABC nuclease have different binding sites has recently been tested by using a series of substrates prepared by treating pBR322 DNA with various modifying reagents including UV light. It was found that binding of photolyase to damaged DNA prior to addition of the nuclease stimulated nuclease activity 2-fold when dimer-containing DNA was used as substrate but had no effect with substrates containing modifications induced by psoralen or *cis*-dichlorodiammineplatinum. We interpret this to mean that, by binding the pyrimidine dimer, photolyase increased the distortion caused by the dimer and thus made it a better substrate for the nuclease.

Registry No. Oligo(dT)$_n$, 25086-81-1; DNA photolyase, 37290-70-3.

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Ultraviolet Resonance Raman Spectra of Insulin and α-Lactalbumin with 218- and 200-nm Laser Excitation†

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Received August 10, 1984

ABSTRACT: Ultraviolet resonance Raman (RR) spectra, with 200- and 218-nm excitation from a H_2-shifted quadrupled Nd:YAG laser, are reported for insulin and α-lactalbumin in dilute aqueous solution, at pH values known to produce differences in the exposure of the aromatic residues to solvent. At 200 nm, the spectra are dominated by tyrosine bands, whose intensity is lowered somewhat in protein conformations in which tyrosine is exposed to solvent. The expected shift in the relative intensities of the components of the $\sim$850-cm^{-1} tyrosine doublet is difficult to discern because the higher energy component shows much greater resonance enhancement and the lower energy component appears as a weak shoulder. The peptide vibrations, amides I, II, and III, are also enhanced at 200 nm. The infrared active amide II mode is particularly prominent, although it is not observed in Raman spectra with visible excitation. In addition, the amide I band is quite broad in the 200-nm RR spectra, and the peak frequency is lower than that seen in visible excitation Raman spectra and is close to the infrared frequency. It appears that 200-nm excitation produces resonance enhancement of the infrared-active components of both amide I and amide II. Excitation at 218 nm enhances tryptophan modes strongly. The 876-cm^{-1} band, assigned to a deformation mode of the five-membered ring, shows a measurable upshift upon exposure of tryptophan to solvent, attributable to N–H hydrogen bonding. The broad band seen at $\sim$1360 cm^{-1} in visible excitation spectra is shown to be a doublet, the two components of which alter their relative intensities upon tryptophan exposure to solvent. When tryptophan is absent in the protein, tyrosine and phenylalanine modes are seen in the 218-nm spectrum.

Since the pioneering studies of Edsall and co-workers in the 1950s (Edsall et al., 1950; Garfinkle & Edsall, 1958a–c; Garfinkle, 1958), there has been continual interest in applying Raman spectroscopy to elucidate the structure of proteins (Lord & Yu, 1970; Spiro & Gaber, 1977). The responsiveness of the vibrational spectrum to molecular geometry gives wide scope to the technique, but with the usual visible light excitation, there are serious limitations of low sensitivity and the overlap of numerous vibrational bands. Accordingly, there is much current interest in the use of ultraviolet excitation, which promises large enhancements of vibrational modes of aromatic residues, and of the peptide bonds, via the resonance Raman (RR) effect (Carey, 1982). The attendant gain in sensitivity and selectivity for these chromophores may permit much more discriminating application of Raman spectroscopy to protein structural problems than has heretofore been possible.

We have recently reported Raman spectra for tryptophan and tyrosine with 218- and 200-nm excitation, obtained with the fourth harmonic of the Nd:YAG laser and a H_2 Raman shift cell (Rava & Spiro, 1984). High quality spectra were obtained in dilute ($\sim$1 mM) aqueous solution, and almost complete selectivity was observed, with tryptophan dominating the 218-nm spectrum and tyrosine dominating the 200-nm spectrum. In this study we examine UV RR spectra, with 200- and 218-nm excitation, for two small proteins, insulin and α-lactalbumin, which have previously been studied by nonresonance Raman (Yu et al., 1972, 1974; Nakanishi, 1974) and other spectroscopic techniques (Muszkat et al., 1984; Sommers & Kronman, 1980). Insulin contains no tryptophan (Trp) but has four tyrosine (Tyr) and three phenylalanine (Phe) residues. Bovine α-lactalbumin has four each of Trp,

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† This work was supported by NSF Grant CHE-8106084. R.P.R. is the recipient of Postdoctoral Research Fellowship NIH 5 F32 GM 09104-02.

0006-2960/85/0424-1861$01.50/0