The N^2-Guanine Adduct but Not the C8-Guanine or N^6-Adenine Adducts Formed by 4-Nitroquinoline 1-Oxide Blocks the 3′–5′ Exonuclease Action of T4 DNA Polymerase†

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ABSTRACT: When *O*-acetyl-4-(hydroxyamino)quinoline 1-oxide (Ac-4HAQO) reacts with double-stranded DNA at 37 °C the major products, N^2-guanine, C8-guanine, and N^6-adenine adducts, are formed in the proportions of 5:3:2, respectively. When the reaction is carried out with single-stranded DNA at 0 °C, the products are found in the ratio 1:7:2. Unique 174-bp DNA fragments were modified in these ways and used as substrates for the 3′–5′ exonuclease activity of T4 DNA polymerase. The results obtained showed that the exonuclease is blocked by the N^2-guanine adduct but not the other two adducts. Interpretation of the cleavage patterns suggested that the enzyme stopped 2 nucleotides before the N^2-guanine adduct. The N^2-guanine adduct lies in the minor groove of the DNA double helix, while the other two adducts are found in the major groove. Apparently, only the former hinders progression of the enzyme.

4-Nitroquinoline 1-oxide (4NQO)[1] produces a wide variety of tumors when administered to experimental animals (Ito, 1981), but it must be metabolically activated before it can react with DNA. 4NQO is reduced to 4-(hydroxyamino)quinoline 1-oxide (4HAQO) by DT-diaphorase (Sugimura et al., 1966) and further metabolized by the action of seryl-tRNA synthetase to the ultimate carcinogen which binds to the DNA by covalent bonding to guanine and adenine moieities (Tada & Tada, 1975). 4NQO can be converted chemically to diAc-4HAQO or Ac-4HAQO, which are able to react directly with DNA to produce the same adducts as those formed in the in vivo reaction (Galiegue-Zouitina et al., 1985). The major products formed and their relative amounts are 3-(*N*^{2}-guanyl)-4-aminoquinoline 1-oxide (N^2-guanine adduct) 50%, *N*-(guan-8-yl)-4-aminoquinoline 1-oxide (C8-guanine adduct) 30%, and 3-(*N*^{6}-adenyl)-4-aminoquinoline 1-oxide (N^6-adenine adduct) 10% (Galiegue-Zouitina et al., 1985, 1986).

A number of studies have shown that mammalian cells that are deficient in repairing UV-damaged DNA are also deficient in repairing 4NQO-damaged DNA (Walker, 1981; Takebe et al., 1972; Zelle & Bootsma, 1980; Tanaka et al., 1980; Collins & Johnson, 1987). Thus, the removal of AQO–purine adducts and UV-induced pyrimidine dimers from DNA appears to proceed by a common pathway. At present nothing is known about the molecular mechanism of the incision and excision processes of AQO-adducted DNA in mammalian cells or what similarity it possesses with the pyrimidine dimer excision process. However, in *Escherichia coli* both pyrimidine dimers and AQO adducts are removed by the uvrABC exinuclease (Sancar & Sancar, 1988). The objective of the present study was to examine the ability of T4 DNA polymerase 3′–5′ exonuclease to hydrolyze AQO-modified double-stranded 5′ end labeled DNA fragments to determine which of the AQO lesions may serve as blocks to the exonuclease at or near the adduct sites. A similar approach has been taken to study the distribution of UV-induced pyrimidine dimers (Doetsch et al., 1985), AAF adducts (Fuchs et al., 1983; Fuchs, 1983), and 8-methoxypsoralen adducts (Sage & Moustacchi, 1987; Boyer et al., 1988) with the T4 DNA polymerase associated exonuclease activity. A study of this kind will allow us to gain insight in the following areas: (i) sequence specificity of AQO adduct formation; (ii) differential disturbance of DNA structure by the three different kinds of AQO adducts as reflected in the behavior of the T4 enzyme toward them.

Materials and Methods

Enzymes and Chemicals. [γ-^{32}P]ATP (specific activity 5209 and 3000 Ci/mmol) was obtained from ICN Biochemicals, Canada, or Amersham, Canada. T4 DNA polymerase, *Eco*RI, and *Bam*HI were purchased from Pharmacia. *Hae*III, T4 polynucleotide kinase, calf intestine alkaline phosphatase, bovine pancreatic DNase I, snake venom phosphodiesterase, and calf spleen phosphodiesterase were obtained from Boehringer Mannheim. 4NQO, calf thymus DNA, and salmon sperm DNA were from Sigma. Poly(dG-dC) and poly(dA-dT) were from Pharmacia. All other chemicals and reagents were of the highest purity available.

Preparation of Ac-4HAQO-Modified Calf Thymus DNA and Deoxyoligonucleotides, Their Enzymatic Hydrolysis to Nucleosides and HPLC Analysis of the Hydrolysates. DiAc-4HAQO was prepared from 4NQO as described before (Panigrahi & Walker, 1986). Ac-4HAQO was prepared by dissolving 1 mg of diAc-4HAQO in 25 μL of dimethyl sulfoxide and adding this to 0.8 mg of dithiothreitol dissolved in 25 μL of dimethyl sulfoxide. After 15 min at room temperature, the preparation was ready to use. Native calf thymus DNA was modified by treating 300 μg of DNA dissolved in 400 μL of 2 mM sodium citrate buffer (pH 7) with 30 μL of the Ac-4HAQO preparation for 30 min at 37 °C. Poly(dG-dC) and poly(dA-dT), 300 μg each, were reacted in an identical fashion. Denatured DNA, 300 μg, was reacted in a similar way but at 0 °C for 30 min. The DNA was denatured

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[1] Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; diAc-4HAQO, *O*,*O*′-diacetyl-4-(hydroxyamino)quinoline 1-oxide; Ac-4HAQO, *O*-acetyl-4-(hydroxyamino)quinoline 1-oxide; 4HAQO, 4-(hydroxyamino)quinoline 1-oxide; AQO, 4-aminoquinoline 1-oxide; AAF, *N*-acetyl-2-aminofluorene; AF, 2-aminofluorene.

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by heating it for 10 min in a boiling water bath and then chilling it in an ice bath. The modified DNAs and deoxyoligonucleotides after purification by ether extraction and alcohol precipitation were dissolved in 300 μL of digestion buffer and hydrolyzed enzymatically to deoxynucleosides according to the method reported previously (Galiegue-Zouitina et al., 1983). HPLC instrumentation and analysis were performed as described before (Panigrahi & Walker, 1986).

Preparation of 5′ ^{32}P End Labeled Double-Stranded 375-bp DNA Fragments. Approximately 350 μg of pBR322 DNA was digested with *Bam*HI and *Eco*RI, and the *Bam*HI–*Eco*RI 375-bp fragment was separated by electrophoresis on a 5% polyacrylamide gel and eluted from the gel by the crush and soak method. The 375-bp fragment was then 5′ end labeled with ^{32}P by using the classical phosphatase–polynucleotide kinase procedure.

Modification of 5′ ^{32}P End Labeled Double-Stranded 375-bp DNA Fragments with Ac-4HAQO. (a) Preparation of AQO-Modified 375-bp DNA Containing N^2-Guanine and C8-Guanine Adducts in the Ratio of 5:3. Approximately 6 μg of ^{32}P-labeled 375-bp fragment was dissolved in 50 μL of 2 mM sodium citrate buffer (pH 7). One microliter of Ac-4HAQO preparation as described above but which had been diluted 4-fold with sodium citrate buffer was added to the DNA solution. The reaction was allowed to proceed at 37 °C for 30 min, after which time the DNA was precipitated with ethanol.

(b) Preparation of AQO-Modified 375-bp DNA in Which the C8-Guanine Adduct Is Greatly Enriched. The same amount of ^{32}P-labeled 375-bp fragment taken above was dissolved in 50 μL of 2 mM sodium citrate buffer (pH 7). It was heated in a boiling water bath for 10 min and then plunged into an ice bath. One microliter of 4-fold diluted Ac-4HAQO solution was added, and the reaction was allowed to proceed at 0 °C for 30 min, after which time the DNA was precipitated with alcohol.

The modified ^{32}P-labeled 375-bp fragments as well as an equal amount of unmodified fragment were each dissolved in 40 μL of *Hae*III digestion buffer and treated with 9 units of the enzyme for 4 h at 37 °C. *Hae*III makes two cuts in the 375-bp fragment. Following electrophoresis of the digest in 5% polyacrylamide gel, the ^{32}P-labeled 174-bp restriction fragment was located as a band on the gel following autoradiography. The band was excised and eluted. Following ethanol precipitation of the ^{32}P 5′ end labeled DNA fragment, it was dissolved in 100 μL of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.

UV Irradiation of Double-Stranded DNA and Digestion with T4 DNA Polymerase (3′–5′) Exonuclease. Unmodified ^{32}P-labeled 174-bp fragments (5-μL droplets from the 100-μL solution described above) were irradiated on ice with a 15-W germicidal lamp (General Electric) emitting most of its radiation at 254 nm. The DNA samples were then diluted with 200 μL of 0.3 M NaOAc and ethanol precipitated. The precipitates were dissolved in 20 μL of assay buffer (33 mM Tris-acetate, pH 7.8, 10 mM $Mg(OAc)_2$, 66 mM KOAc, 0.5 mM DTT, and 0.1 mg/mL BSA) and incubated with 0.5 μL (10 units/μL) of T4 DNA polymerase for 30 min at 37 °C. Since this amount of enzyme was found to degrade completely the unmodified DNA, it was used in all the other digestion reactions. The reaction was stopped by addition of 1 μL of salmon sperm carrier DNA (1 μg) and 0.3 M NaOAc (200 μL) followed by extraction with buffer-saturated phenol and then with chloroform–isoamyl alcohol (24:1) and ethanol precipitation.

Table I: Relative Amounts of AQO Adducts Formed in the Reaction between Ac-4HAQO and DS DNA at 37 °C or SS DNA at 0 °C

	amount of AQO adduct as a percent of the three major adducts		
reaction condition	N^2-guanine	C8-guanine	N^6-adenine
ds DNA, 37 °C	47	30	23
ss DNA, 0 °C	8	72	20

Digestion of Ac-4HAQO-Modified 174-bp DNA with T4 DNA Polymerase (3′–5′) Exonuclease. DNA samples (5 μL and 20 000 cpm Cerenkov) were precipitated and dissolved in 20 μL of assay buffer and incubated with 5 units (0.5 μL) of T4 DNA polymerase for 1 h at 37 °C. The reaction was terminated, and the samples were processed as described above.

Piperidine Digestion of Ac-4HAQO-Modified 174-bp DNA. DNA samples (5 μL) modified in the two different reaction conditions were dissolved in 1 M piperidine (100 μL) and heated for 30 min at 90 °C. Piperidine was removed by using a Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, NY).

DNA Base-Specific Sequence Reactions. Maxam–Gilbert base-specific chemical degradation reactions for A+G and C+T were performed (Maxam & Gilbert, 1980). The G-specific reaction was obtained by treating Ac-4HAQO-modified DNA with hot piperidine.

Gel Electrophoresis. DNA samples were dissolved in 2 μL of loading buffer heated to 90 °C for 4 min and quickly chilled in an ice bath. The samples were loaded on to prerun, denaturing 8% polyacrylamide and 7 M urea gel and electrophoresed at 1500 V for 1.5 h. After electrophoresis, the gel was dried and autoradiographed by using an intensifying screen at −70 °C.

Densitometry. Autoradiograms were scanned with a LKB ultro scan XL laser densitometer to produce profiles from which the relative intensity of each band was measured.

Results

Ac-4HAQO was reacted at 37 °C with native calf thymus DNA, poly(dG-dC), and poly(dA-dT) or with denatured calf thymus DNA at 0 °C. The modified DNAs or polynucleotides were degraded to nucleosides and analyzed by HPLC (Figure 1). Identification of the adducts was made by comparison of the HPLC profiles with that reported previously (Galiegue-Zouitina et al., 1986). The reaction with poly(dA-dT), panel A, produced the expected adenine adduct, while the reaction with poly(dG-dC), panel B, produced the two expected guanine adducts. The reaction with native DNA, panel C, or denatured DNA, panel E, produced both the guanine and adenine adducts. Panel D shows the profile produced when unmodified DNA is put through the analytical procedure. A fourth peak that elutes soon after the N^{2}G adduct is seen in the reaction with native DNA, denatured DNA, or poly(dG-dC). It corresponds in elution position to AQO previously identified by Galiegue-Zouitina et al. We do not detect any incompletely hydrolyzed material as was the case previously (Panigrahi & Walker, 1986; Galiegue-Zouitina et al., 1986), probably because the present reaction was carried out on a much smaller scale. For the reaction with native DNA, the major product was the N^2-guanine adduct as found previously (Galiegue-Zouitina et al., 1986; Panigrahi & Walker, 1986). In contrast, when the reaction was carried out with single-stranded DNA at 0 °C, the C8-guanine adduct was formed predominantly. The relative amounts of the various adducts, recorded in Table I, were estimated by measuring the area of the peaks in the HPLC profiles and correcting for the dif-

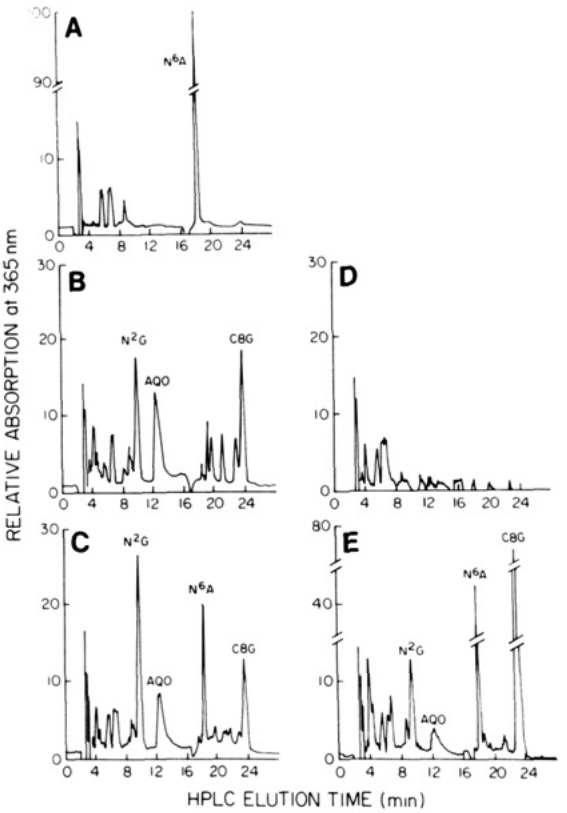

FIGURE 1: HPLC elution profiles of enzymatically hydrolyzed, Ac-4HAQO-modified DNA and polynucleotides. (A) Modified poly(dA-dT). (B) Modified poly(dG-dC). (C) Modified native DNA. (D) Unmodified DNA. (E) Modified denatured DNA. The conditions for modifying the DNA with Ac-4HAQO are described in the text. In each case a 100-μL sample of hydrolysate was injected into the HPLC column. Abbreviations: AQO, 4-aminoquinoline 1-oxide; C8G, *N*-(deoxyguanosin-8-yl)-4-aminoquinoline 1-oxide; N^6A, 3-(deoxyadenosin-N^6-yl)-4-aminoquinoline 1-oxide; N^2G, 3-(deoxyguanosin-N^2-yl)-4-aminoquinoline 1-oxide.

ference in extinction coefficients of the adducts (Galiegue-Zouitina et al., 1984, 1986).

When double-stranded DNA was modified by treatment with Ac-4HAQO and used as a substrate for T4 DNA polymerase 3′–5′ exonuclease, the digestion pattern shown in Figure 2 was obtained. The pattern shows a G ladder that is displaced upward from the Maxam–Gilbert G ladder by 3–4 nucleotides. There is no evidence in this profile, or in other overexposed profiles, of an A ladder. Thus, the exonuclease action has been blocked by AQO-modified guanines and at a position several nucleotides in front of the modified guanines. By way of comparison, the action of the T4 enzyme on UV-irradiated DNA was examined, and the digestion profile obtained was the same as that reported by others (Doetsch et al., 1985). The fragments containing pyrimidine dimers migrated with an electrophoretic mobility of a DNA fragment approximately 1.5–2 bases longer than the corresponding Maxam–Gilbert sequencing reaction product (data not shown). After a correction was made for the presence of the additional nucleoside in the dimer structure, the absence of a 3′-phosphate, and the fragment length, it could be concluded that the exonuclease action is halted immediately in front of the pyrimidine dimer with no preceding nucleotides (Doetsch et al., 1985).

Since the AQO-modified DNA used as a substrate in the experiment above contained the N^2- and C8-quanine adducts in similar proportions, it was not possible to tell which of these or both, was responsible for halting the enzyme's progress. An answer to this question was made possible by employing a

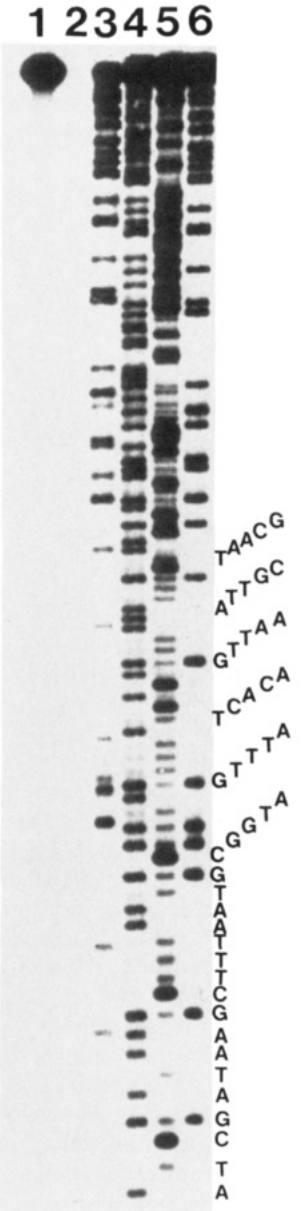

FIGURE 2: Electrophoretic pattern of products formed by the action of T4 DNA polymerase on the 174-bp ds DNA fragment modified by Ac-4HAQO. (Lane 1) Nonmodified DNA; (lane 2) nonmodified DNA plus enzyme; (lane 3) modified DNA plus enzyme; (lane 4) Maxam–Gilbert G+A reactions; (lane 5) Maxam–Gilbert C+T reactions; (lane 6) modified DNA plus piperidine treatment.

DNA substrate in which the AQO modification was predominantly the C8-guanine form by treating single-stranded DNA with Ac-4HAQO at 0 °C. The AQO-modified single-stranded DNA (375 nucleotides) renatured readily because it was found that when it was treated with *Hae*III, it yielded the expected three fragments, and the 5′ ^{32}P labeled 174-bp fragment could therefore be used as a substrate for the T4 DNA polymerase exonuclease. Figure 3 shows the digestion patterns obtained when this substrate (lane 2) as well as an equal amount of the other AQO-modified DNA in which the N^2-guanine adduct predominated (lane 1) was treated with the T4 enzyme. In lane 2 the G ladder is very faint, and there is much less DNA in this lane compared to that in lane 1. The interpretation of this result is that the C8-guanine adduct does not block the progress of the exonuclease, so that most of the DNA substrate is completely digested. The faint G ladder is due to the small number of N^2-guanine adducts present. Confirmation of this concept was obtained by determining the amount of ^{32}P made

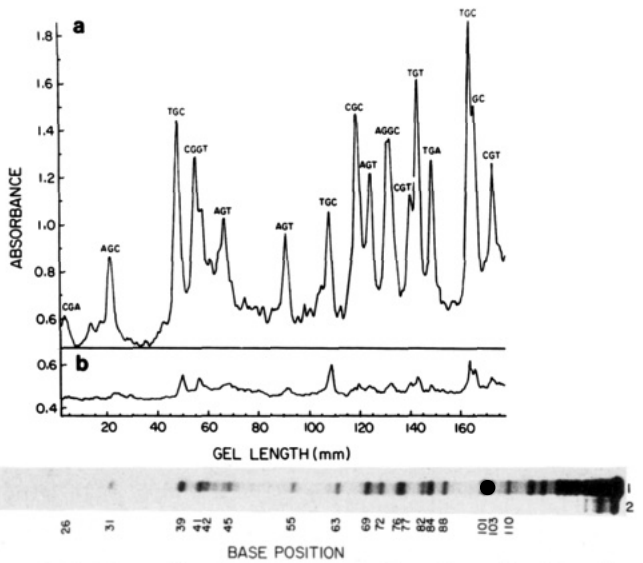

FIGURE 3: Electrophoretic patterns of products formed by the action of T4 DNA polymerase on the 174-bp DNA fragment modified by Ac-4HAQO. (Lane 1) DNA was modified in its double-stranded form at 37 °C; panel a is the densitometric tracing. (Lane 2) DNA was modified in its single-stranded form at 0 °C; panel b is the densitometric tracing (see Materials and Methods for details). The guanines are numbered starting from the 5′ end of the DNA fragment. The nucleotide sequence of the trinucleotide containing the guanines is given above each peak in panel a.

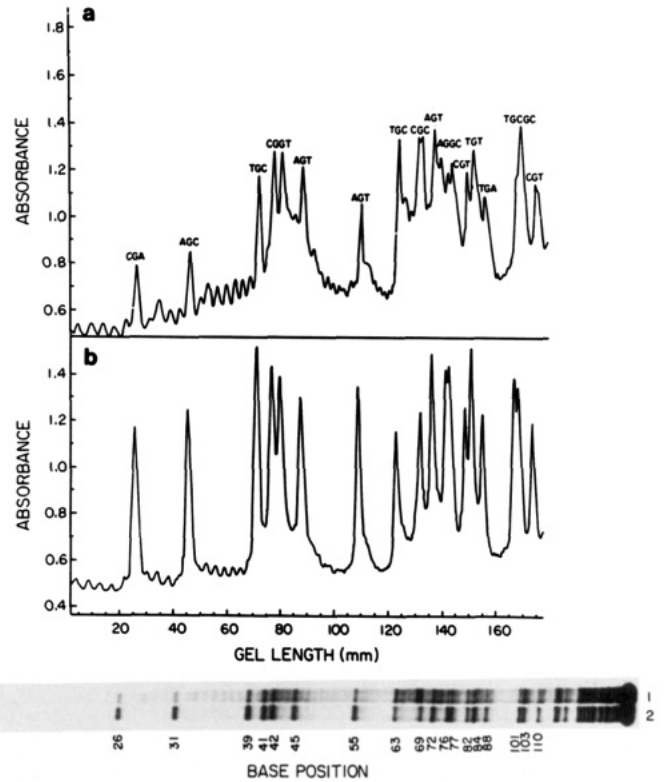

FIGURE 4: Electrophoretic patterns of the products formed by the action of piperidine on the 174-bp DNA fragment modified by Ac-4HAQO. (Lane 1) DNA was modified in its double-stranded form at 37 °C; panel a is the densitometric tracing. (Lane 2) DNA was modified in its single-stranded form at 0 °C; panel b is the densitometric tracing (see Materials and Methods for details). Other details as in Figure 3.

ethanol soluble after exonuclease digestion. Additional evidence for this concept was obtained by treating equal-sized samples of both modified DNA substrates with hot piperidine, which will cause the formation of single-strand breaks at sites of C8-guanine adducts (Galiegue-Zouitina et al., 1985). In Figure 4, lane 1 contains the DNA that had been modified at 37 °C in its double-stranded form; lane 2 contains the DNA that had been modified at 0 °C in its single-stranded form. The intensities of the bands of the G ladder in lane 2 are greater than those in lane 1, and this is indicative of the greater number of C8-guanine adducts per DNA molecule in the substrate prepared from single-stranded DNA at 0 °C. Since piperidine reveals the location of C8-guanine adducts, the enzyme is revealing the location of the N^2-guanine adducts. The influence of neighboring nucleotides on the extent of formation of the C8-guanine adduct is apparent in the profile shown in Figure 3a. However, the rules governing this specificity appear complex. For example, one TGC sequence shows an average reactivity, while two other TGC sequences show reactivities that are about 1.5–2 times the average. Similarly, the formation of N^2-guanine adducts is clearly influenced by neighboring nucleotides (Figure 4a), but again the relationship is complex.

Discussion

Ac-4HAQO reacts with DNA to form AQO adducts at the N^2 and C-8 positions of guanine and the N^6 position of adenine. Our results indicate that only the N^2-guanine adduct blocks the 3′-5′ exonuclease activity of T4 DNA polymerase. The enzyme-generated DNA fragments migrate as if they were 3–4 nucleotides longer than the corresponding guanine-specific chemical reaction. When the size of the enzyme-generated fragment is less than 30 nucleotides, the mobility difference corresponds to an additional 4 nucleotides. Between fragment sizes of 30 and 50 nucleotides the difference is 3.5 nucleotides. For a fragment size of more than 50 nucleotides the mobility difference is 3 nucleotides. Previously it had been demonstrated that for polydeoxynucleotides containing 30 nucleotides

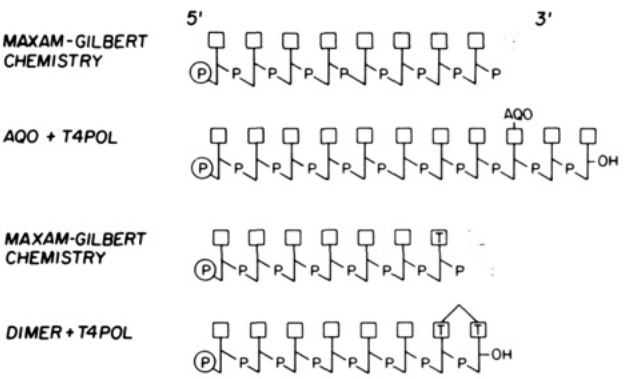

FIGURE 5: Scheme for comparison of T4 DNA polymerase digestion product with Maxam–Gilbert chemical degradation product.

or less and differing solely by the presence or absence of a terminal phosphate, the polynucleotide lacking the terminal phosphate migrated in a polyacrylamide gel as if it were one nucleotide longer and the mobility difference decreased to zero as the size of the polynucleotides became larger than 50 (Tapper & Clayton, 1981). The T4 enzyme-generated DNA fragments contain a 3′-OH group, whereas the chemically generated DNA fragments have a phosphate at the 3′ position. It is concluded, therefore, that the structure of the AQO-modified DNA fragment remaining after the action of T4 polymerase contains 2 nucleotides, 3′ to the adduct, and the last nucleotide is terminated with a 3′-hydroxyl group. This structure is illustrated in Figure 5 along with the corresponding Maxam–Gilbert product. For comparison the product formed when T4 polymerase is stopped by a pyrimidine dimer is also shown. A similar result was obtained in a study of the exonuclease action of T4 DNA polymerase on DNA fragments

that had been modified with *N*-acetoxy-2-(acetylamino)-fluorene (Fuchs et al., 1983). However, in another publication it had been concluded that the structure of the enzyme-generated fragments contained only one additional nucleotide on the 3′ side of the guanine–AAF residue (Fuchs, 1983). This conclusion was reached because it was assumed that the enzyme-generated fragments migrated as if they were 3 rather than 4 nucleotides longer than the chemically produced fragments.

The C8 position of guanine and the N^6 position of adenine are located in the major groove of the DNA double hexlix, while the N^2 position of guanine lies in the minor groove. The inhibitory effect of the N^2-guanine adduct might have a steric basis or it might be due to a distortion of the DNA double helix that is sensed by the T4 enzyme. The C8 guanine AAF adduct blocked the action of the T4 enzyme, but the deacetylated C8 guanine AF adduct did not (Bichara & Fuchs, 1985). Previously it had been demonstrated that AAF adducts induced a major conformational change (Fuchs et al., 1976), while AF adducts affected the DNA conformation only slightly (Daune et al., 1981). These findings provide a basis for the differential effects of the AAF and the AF adducts on the action of the T4 enzyme. By analogy, it also suggests why the C8 guanine AQO adduct, which is even smaller in size than the AF adduct, fails to halt the progress of the T4 enzyme.

The action of other enzymes is also halted by the presence of guanine adducts in DNA. T4 phage pyrimidine dimer DNA glycosylase was inhibited in its ability to incise at thymine dimer sites if guanine–AQO adducts were present in the DNA (Duker & Merkel, 1986). The methyl accepter capacity of DNA in the presence of DNA cytosine 5-methyltransferase was reduced in proportion to the extent that it had been modified by AAF (Pfohl-Leskowiez et al., 1981). An interesting feature of these studies is that they provide evidence for the processive mechanism of the two enzymes.

If one assumes the operation of essentially error free DNA repair mechanisms, the ability of adducts in DNA to be recognized and removed by DNA repair systems will be reflected ultimately in the mutagenicity of the adduct-forming agent. The C8-guanine adduct of AAF was rapidly removed from rat liver in vivo, whereas the N^2-guanine adduct remained persistently bound to DNA (Kriek, 1972). The AAF lesions induced frameshift mutations almost exclusively, while AF lesions led to a preponderance of G to T transversions (Bichara & Fuchs, 1985). 4NQO preferentially induced G–C to A–T transitions at the rII locus in bacteriophage T4 (Ishizawa & Endo, 1970, 1971). Others have found that 4NQO also yielded G–C to A–T transitions preferentially in the lac I gene of *E. coli* (Coulondre & Miller, 1977; Miller et al., 1977). Currently it is not known which of the AQO adducts is responsible for the induction of the transition mutation. It should be possible to determine the responsible adduct by examining the mutations formed in transfected plasmids or shuttle vectors that carry both C8-guanine and N^2-guanine adducts or essentially only the C8-guanine adduct.

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