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Detection of thymine [2+2] photodimer repair in DNA: selective reaction of $KMnO_4$

Danaboyina Ramaiah[+], Troels Koch[1], Henrik Ørum[1] and Gary B. Schuster*

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400, USA and
[1]PNA Diagnostics A/S, Rønnegade 2, DK-2100 Copenhagen, Denmark

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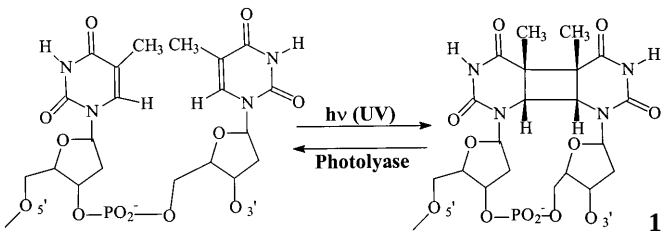
ABSTRACT

The specific reaction of potassium permanganate with thymine in single-stranded DNA was employed to analyze thymine [2+2] dimer repair in DNA and in DNA/peptide nucleic acid hybrid duplexes. This simple and highly sensitive chemical assay is convenient for monitoring repair of thymine dimers in oligonucleotides.

INTRODUCTION

Exposure to UV light damages DNA and causes mutations. The major lesions formed in DNA are pyrimidine dimers generated as a result of [2+2] photocycloaddition between adjacent bases on a DNA strand (1 and references therein, 2). Repair of these dimers (regeneration of the monomeric bases) has been observed to occur photochemically by direct irradiation with UV light, by photosensitized electron transfer (PET) and by an enzymatic reaction catalyzed by photolyase (3–7). These processes are illustrated in equation **1** for adjacent thymines. We recently discovered the photochemical formation of thymine dimers in peptide nucleic acids (PNA) and PNA/DNA hybrid duplexes (K.O'Shea, D.Ramaiah, T.Koch, H.Orum and G.B.Schuster, submitted) and their photochemical repair by PET and by reaction with photolyase (D.Ramaiah, T.Koch, H.Orum and G.B.Schuster, submitted).



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One of the major challenges encountered in investigation of these photolesions in DNA is detection and localization of the site of thymine dimer repair. Development of analytical methods for convenient and reliable analysis is especially important because these lesions may lead to skin cancer (8). Currently, the presence and location of thymine dimers in DNA is ordinarily determined by enzymatic digestion with T4 DNA polymerase. Reaction starts at the 3′-end of the DNA strand and cleavage does not usually proceed past the site of dimerization (9,10). This method of analysis is cumbersome and error-prone. The results obtained are sensitive both to the source of the enzyme and to the reaction conditions. HPLC provides a second general analytical method for determination of thymine dimer repair. In this case, an experimental sample is compared with specifically synthesized authentic DNA oligomers containing dimers at know locations (10). However, this method requires relatively large quantities of material and it is inapplicable if the sequence is unknown. Our work on the photodimerization of thymines and their repair in PNA/DNA hybrid duplexes prompted us to seek a new, selective and convenient analytical method for monitoring repair of thymine photodimers in oligonucleotides.

We first investigated the effect of thymine dimerization on the migration rate of ^{32}P-5′-end-labeled DNA oligomers by PAGE. We found that dimer-containing strands migrate more slowly than strands containing monomeric thymines, presumably a consequence of a 'kink' caused by the dimer. This method is convenient and sensitive, however, it is restricted to DNA oligomers containing no more than 19 nt. In longer sequences the difference in mobility between the dimer-containing and the unmodified strand is too small to be reliably distinguished. Consequently, we sought an alternative, more general method for analysis of thymine [2+2] dimer repair in DNA.

Maxam and Gilbert's chemical method of sequencing DNA relies upon chemical modification specific to a base followed by selective piperidine-induced β-elimination to cleave the sugar–phosphate backbone only at the modified sites (11). Hydrazine is used to give selective cleavage at both cytosines and thymines and osmium tetraoxide (OsO_4) and potassium permanganate ($KMnO_4$) are used for specific reaction with thymine under defined experimental conditions (12–14).

The utility of $KMnO_4$ as an analytical reagent has been demonstrated previously with its use to detect modifications to the structure or conformation in B-DNA by intercalative binding of ethidium (15) and also for the analysis of various PNA invasion complexes (16,17). The reaction of $KMnO_4$ with thymine is known to involve specific *cis*-dihydroxylation of the 5,6 double

*To whom correspondence should be addressed. Tel: +1 404 894 3300; Fax: +1 404 894 7466; Email: gary.schuster@cos.gatech.edu

[+]Present address: Photochemistry Research Unit, Regional Research Laboratory (CSIR), Trivandrum 695 019, India

Table 1. DNA and PNA sequences

DNA(1)	5'-C G C G T T G C G C-3'
DNA(3)	5'-C G C G {**T T**} G C G C -3'
DNA(4)	5'-T G C A C G C A T T G C A G G T A G C-3'
DNA(5)	3'-A C G T G C G T A A C G T C C A T C G-5'
DNA(6)	5'-T G C A C G C A {**T T**} G C A G G T A G C -3'
DNA(7)	5'-C A C G C A T T G C A C G C G T T G C G C A-3'
DNA(8)	3'-G T G C G T A A C G T G C G C A A C G C G T-5'
DNA(9)	5'-C A C G C A {**T T**} G C A C G C G T T G C G C A-3'
PNA(3)	H-Lys-Lys-A C G T G C G T A A C G T C C A T C G-NH_2

bond (18,19). We reasoned that the [2+2] dimer of thymine might be inert to reaction with $KMnO_4$, since the 5,6 double bonds are consumed in the cycloaddition.

Herein we report a versatile, simple and extraordinarily sensitive assay for thymine [2+2] dimer repair in DNA. This method makes use of the specific reaction of $KMnO_4$ with thymines in single-stranded DNA. It is applicable to the analysis of oligonucleotides containing multiple dimers and reveals their precise location even in the presence of undimerized thymines. Further, we found that thymines in a PNA/DNA hybrid duplex are not protected from reaction with $KMnO_4$, as they are in duplex DNA.

MATERIALS AND METHODS

Preparation and purification of PNA and DNA containing a thymine dimer

The PNA oligomers were prepared, purified and characterized as previously described (20). DNA oligomers were purchased from Midland Certified Reagents. DNA oligomers containing thymine dimers were prepared by irradiation of deoxygenated (Argon) room temperature aqueous solutions (1 ml, 100 µM) for 4 h with a 1000 W Hg/Xe lamp through an Oriel Corp. 280 nm cut-off filter. The irradiated solution was concentrated on a Speedvac and the mixture was separated by reversed phase HPLC (Rainin Microsorb-MV 18; 4.6 × 50 × 250 mm, 300 Å) with a linear gradient of triethylammonium acetate (0.1 mM, pH 7), water and acetonitrile. The product collected was the purified oligomer containing a TT dimer.

Analysis of [2+2] thymine dimer repair by photolyase

The oligonucleotides were labeled with ^{32}P at the 5′-end using standard techniques (21). The radiolabeled oligonucleotide (2500 c.p.m) was mixed with various amounts of complementary PNA and DNA in 9 µl 10 mM phosphate buffer containing enzyme assay buffer (50 mM Tris–HCl, 10 mM NaCl, 1.7 mM DTT and 1 mM EDTA, pH 7.4) (22,23). Hybridization was carried out by heating to 90°C and then cooling to room temperature for 2 h. Photolyase was added (1 µl 1 µM solution), the samples incubated in the dark for 30 min and then irradiated for 7 min in a Rayonet (350 nm lamps) photoreactor at ~15–20°C. Denaturation was accomplished by adding 1 µl (100 µM) cold DNA and heating at 90°C for 3 min followed by cooling on ice.

Sequencing by $KMnO_4$

The T sequencing by means of $KMnO_4$ used a modified version of the standard procedure. Oligomer samples were added to 1 µl 0.5 mM calf thymus DNA, 1 µl 100 mM phosphate buffer and 7.5 µl water and mixed by vortexing for 5 s and then centrifuged for 5 s at 12 000 r.p.m. A freshly prepared solution of $KMnO_4$ (0.5 µl, 0.5 M) was added to the samples. The reaction proceeded for 45 s and was then quenched by adding DNA precipitating buffer. The precipitated DNA was washed with 80% ethanol, dried and subjected to piperidine treatment (100 µl 1 mM piperidine for 1 h at 90°C). The samples were added to loading buffer, analyzed by 20% polyacrylamide gel electrophoresis (19:1 acrylamide:bisacrylamide), followed by autoradiography. The gel was run with TBE buffer containing 89 mM Tris–borate and 2 mM EDTA (pH 8.3) at 1500 V for 2–3 h.

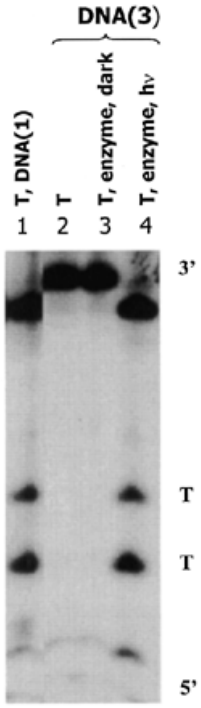

Figure 1. Autoradiogram demonstrating the difference in reactivity with $KMnO_4$ of: lane 1, **DNA(1)**; lanes 2–4, **DNA(3)**. Lanes 3 and 4 contained photolyase (100 nM in enzyme assay buffer) and lane 4 was irradiated at 350 nm for 7 min at ~15–20°C.

RESULTS AND DISCUSSION

The structures of the DNA and PNA sequences examined in this work are shown in Table 1. We generated photodimers in the synthetic oligonucleotides and characterized them by chemical, spectroscopic and enzymatic methods. Irradiation ($\lambda > 280$ nm) of **DNA(1)**, **DNA(4)** or **DNA(7)** results in an absorbance decrease at 260 nm that is typically characteristic of the formation of thymine photodimers (24,25). The product mixture was separated in each case by HPLC and the major products were collected. The characterization of products **DNA(3)**, **DNA(6)** and **DNA(9)** as containing *cis*,*syn*-[2+2] thymine dimers was carried out by gel mobility assay, by their repair with photolyase, which is specific for that one stereoisomer, and finally by reaction with $KMnO_4$.

Figure 1 shows the sensitivity and selectivity of the $KMnO_4$ assay method for thymine dimer repair clearly and convincingly. Lane 1 is the usual Maxam and Gilbert $KMnO_4$ T sequencing experiment for **DNA(1)** which had been 5′-end-labeled with ^{32}P.

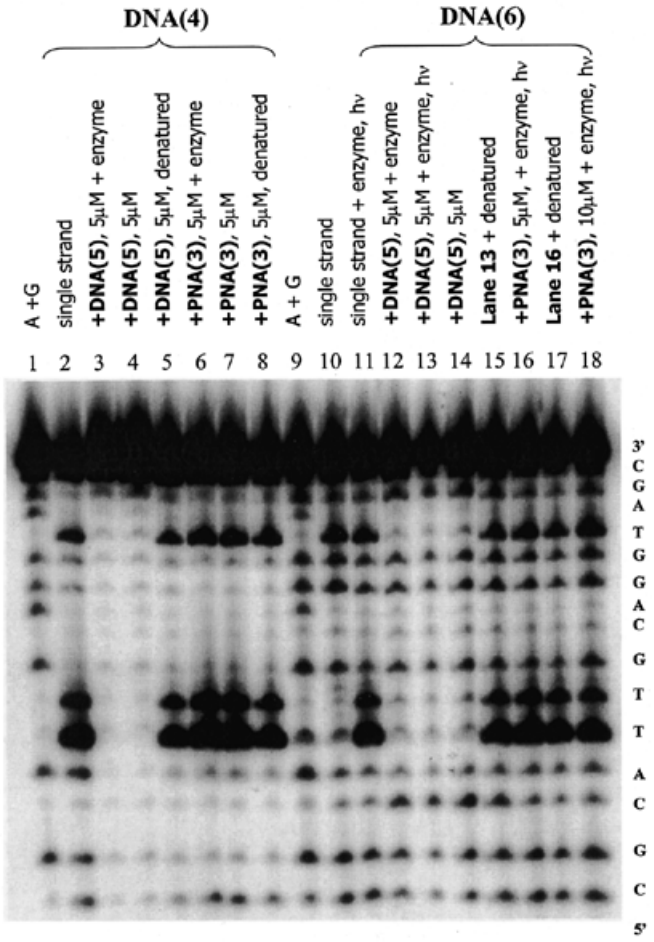

Figure 2. Autoradiogram demonstrating repair of the thymine photodimer in **DNA(6)** by DNA photolyase. Samples in lanes 3–8 and 12–18 contained photolyase (100 nM in enzyme assay buffer). The samples in lanes 11 and 13–18 were irradiated at 350 nm for 7 min at ~15–20°C.

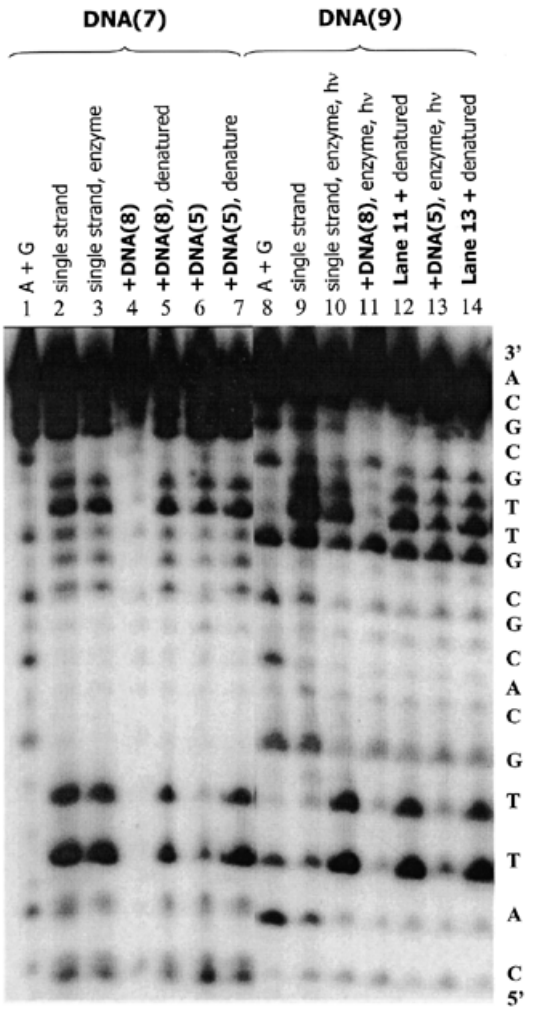

Figure 3. Autoradiogram demonstrating repair of the thymine photodimer in **DNA(9)** by DNA photolyase. The concentration of complementary strand was 5 μM in all cases. The samples in lanes 3–7 and 11–14 contained photolyase (100 nM in enzyme assay buffer). The samples in lanes 10–14 were irradiated at 350 nm for 7 min at ~15–20°C.

As expected, the two thymines in this oligonucleotide are clearly revealed in the autoradiogram as strand breaks following piperidine treatment. Lane 2 of this figure shows the result of an identical treatment of **DNA(3)** with $KMnO_4$ and piperidine. Quite obviously, there is no strand cleavage at either of the thymines of the dimer. Comparison of the intact **DNA(1)** and **DNA(3)** strands (top of the gel) reveals a faster migration rate for **DNA(1)**. Lanes 3 and 4 confirm these results. Photolyase was added to the sample in lane 3 and the solution was kept in the dark. There is no repair of the thymine dimer and there is no cleavage at the thymines. When this solution was exposed to light (λ >350 nm, lane 4) the dimer was efficiently repaired. The repaired oligonucleotide migrates with **DNA(1)** and cleavage at each of the thymines is again observed. Clearly, *cis,syn*-[2+2]-thymine dimers are not cleaved when DNA oligomers are treated with $KMnO_4$ and piperidine.

The utility of the $KMnO_4$ assay for thymine dimer repair is further revealed by the experiments shown in Figure 2. **DNA(4)** is a 19mer that contains an isolated T and a single TT step. Its complement is **DNA(5)** (T_m of duplex = 57°C) and it forms a hybrid PNA/DNA duplex with **PNA(3)** (T_m = 67°C). **DNA(6)** was formed by UV irradiation of **DNA(4)** and contains a single thymine dimer (*vide infra*). It too forms duplexes with **DNA(5)** and **PNA(3)** (T_m = 53 and 57°C respectively). Lanes 1 and 2 of Figure 2 are the usual Maxam–Gilbert A+G and T sequencing experiments for **DNA(4)**. The three thymines are each clearly revealed as strand breaks following treatment with $KMnO_4$ and piperidine (lane 2). In comparison, lane 10 shows the result of treatment of **DNA(6)** with $KMnO_4$ and piperidine. Cleavage at the isolated T is still evident, but there is almost no cleavage at the TT dimer. Inspection of the **DNA(6)** lanes in Figure 2 shows 'background' cleavage at all guanines. This is a consequence of unavoidable oxidative damage during its preparation by UV irradiation. Confirmation that **DNA(6)** contains a *cis,syn*-TT dimer comes from its repair by photolyase (compare lanes 10 and 11).

Formation of the **DNA(4)/DNA(5)** duplex protected all three thymines in **DNA(4)** from attack by $KMnO_4$ (Fig. 2, lane 3). As expected, denaturation of the duplex restores thymine sensitivity (lane 5). In contrast, formation of the hybrid duplex with **PNA(3)** does not afford any protection to the thymines of **DNA(4)** (lane 7). The TT dimer was also repaired by photolyase in duplex DNA,

but this was not revealed by treatment of the duplex with $KMnO_4$ and piperidine until the duplex had been denatured (lane 15). The TT dimer in the PNA/DNA hybrid duplex was also repaired by photolyase (compare lanes 10 and 16), but in this case denaturation was not required to reveal the repaired thymines (compare lanes 16 and 17).

Further demonstration of the utility of the $KMnO_4$ assay is revealed in Figure 3, which shows analyses of **DNA(7)** and **DNA(9)**. **DNA(7)** (lanes 1–7) gives results analogous to those for **DNA(4)**. Both of the TT steps in the oligomer were revealed by $KMnO_4$ treatment (lane 2) and all thymines were protected (lane 4) in the fully complementary duplex with **DNA(8)**, but only the thymines in the double-strand region were protected in the partial duplex with **DNA(5)**. UV irradiation of **DNA(7)** should generate three products: dimerization of the 5′-end thymines; dimerization of the 3′-end thymines; an oligomer containing both pairs of thymines as dimers. Analysis of the solution from irradiation of **DNA(7)** by HPLC showed the expected mixture. One of the products, **DNA(9)**, was isolated, purified and analyzed (lanes 8–14).

Treatment of **DNA(9)** with $KMnO_4$ showed that it is the oligomer in which the 5′-thymines had been dimerized (lane 9). Treatment of **DNA(9)** with photolyase repaired the dimer (lane 10) and, as in the previous case, repair of the dimer in duplex **DNA(9)**/**DNA(8)** and in partial duplex **DNA(9)**/**DNA(5)** was revealed only after denaturation (lanes 12–14). Clearly, the $KMnO_4$ assay is extraordinarily valuable for the analysis of repair in mixtures of oligomers containing thymine dimers.

CONCLUSION

The selective reaction of $KMnO_4$ with thymines in single-stranded DNA has been a useful tool since its discovery by Hayatsu and Ukita (19). The primary product formed in its reaction with thymidine is 5,6-dihydroxy-5,6-dihydrothymidine. This follows from the well-known use of $KMnO_4$ for conversion of olefins to yield *cis*-diols. Thymine [2+2] dimers do not have an olefinic double bond and consequently they will not react or will react much more slowly with $KMnO_4$ than thymine. Our results show that this difference in reactivity provides a convenient and reliable method to detect thymine dimer repair in DNA oligomers. This assay will be useful in the examination of photolyase and in the assessment of the mechanism of thymine dimer repair.

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