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^{31}P NMR characterization of terminal phosphates induced on DNA by the artificial nuclease 'Mn-TMPyP/$KHSO_5$' in comparison with DNases I and II

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ABSTRACT

Phosphorus-31 NMR has been applied to the characterization of terminal phosphates on fragments of calf thymus DNA induced by three different nuclease systems: DNase I, DNase II and the artificial nuclease 'Mn-TMPyP/$KHSO_5$'. In this last case, the oxidative damage to deoxyribose leads to two monophosphates esters (at the 3′ and 5′ ends) on both sides of the cleavage site. This method constitutes a promising approach to visualise the phosphate termini generated in DNA or RNA cleavage by cytotoxic drugs or chemical nucleases and provides a novel insight into the molecular aspects of their mechanism of action.

INTRODUCTION

Nuclear magnetic resonance spectroscopy is a powerful method to elucidate the structure and dynamics of DNA fragments (1, 2). While most of the studies are performed with high-field proton 1D- or 2D NMR, phosphorus-31 NMR is still convenient to obtain informations on nucleic acids (2). ^{31}P chemical shifts and ^{1}H-^{31}P coupling constants provide valuable informations on the phosphate ester backbone conformation, but little direct information is available on the sugar phosphate termini. However, there are ^{31}P resonances having pH dependent chemical shifts downfield with respect to the phosphodiester chemical shifts. These downfield ^{31}P signals can be observed for low molecular weight DNA (3). They are best correlated with phosphate monoester termini. Quite surprisingly, only a limited attention was paid to these signals even after the extensive studies on nucleotides (4). Until recently, there were very few ^{31}P data about structural features of nucleic acid fragment ends. This was mainly because most of the studies in this area have been restricted to the use of synthetic oligodeoxyribonucleotides with free 3′ and 5′ terminal hydroxyl groups. Thus few data can be found into the literature:

(i) procedures for determination of thermodynamic values for the duplex formation of the octamer $[d(GGAATTCC)]_2$ using spectroscopic methods, including ^{31}P NMR studies on phosphodiester or terminal phosphate groups, have demonstrated that attachement of 5′ phosphate groups can significantly stabilize the duplex, while addition of 3′ phosphate groups has only a slight stabilizing effect (5),

(ii) the appearance of terminal phosphates downfield from the backbone ^{31}P resonances have also been observed previously in $tRNA^{Phe}$ (6) and 5S rRNA (7).

In our case, the ^{31}P NMR characterization of terminal phosphates may improve our understanding of how phosphate ending groups are left after enzymatic and/or chemical breaks of DNA, especially if these fragments have to be used as substrates for ligation or for any other DNA repair enzymes.

During the last decade, in addition to the great research efforts on the evaluation of naturally occurring DNA cleaving agents (8), it should be noted the interest in the design and synthesis of model compounds that can specifically recognize and cut DNA. Among these chemical DNA cleavers (9–13), nuclease activities have been reported for (metallo)porphyrin derivatives after photoactivation (14–16) or after being activated by molecular oxygen in the presence of a reducing agent (17,18) or an oxygen atom donor like iodosylbenzene (19). Previous works in our laboratory (20–24) have described the DNA cleavage efficiency of the cationic manganese porphyrin, *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrinatomanganese^{III} pentaacetate (Mn-TMPyP), associated to potassium monopersulfate ($KHSO_5$). Dabrowiak *et al.* have shown that this system can constitute an useful tool for DNA footprinting (25). When covalently linked to an intercalating agent, these cationic metalloporphyrin derivatives exhibit a cytotoxic activity (26, 27) and are also able to cleave DNA (28).

Preliminary studies on the mechanism of DNA cleavage by Mn-TMPyP have shown that the sugar C1′-H bond is the main target of the active species (23,24): hydroxylation at this site leads, after two β-eliminations, to the release of a free base, of 5-methylene-2-furanone (5-MF) as sugar residue and of two postulated terminal 3′- and 5′-phosphate monoesters. Whereas

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the free base and 5-MF have been clearly identified by HPLC methods (23,24), here we report our attempts to identify the effective release of two types of phosphate monoesters (3′ and 5′) using ^{31}P NMR spectroscopy. The work has been carried out on CT DNA. The identification of the ^{31}P NMR resonances of 3′ and 5′ terminal phosphate monoesters has been facilitated by the use of DNase I and DNase II.

MATERIALS AND METHODS

DNA solutions

Stock solution of calf thymus (CT) DNA (Sigma) was prepared in 100 mM NaCl, 160 mM arseniate pH 7.2 buffer and sonicated for 6 hours. Exact DNA concentration (in bases) was determined using an extinction coefficient $\epsilon_{260nm} = 6600\ M^{-1}cm^{-1}$.

Metalloporphyrin cleavage of DNA

To 11 ml of 2.3 mM DNA stock solution, 4 ml of a 175 μM solution of Mn-TMPyP in 100 mM NaCl, 15 mM arseniate pH 7.2 buffer were added. Mn-TMPyP was prepared as previously described (20) and its exact concentration in solution was determined by using the following coefficient: $\epsilon_{465nm} = 10^5\ M^{-1}cm^{-1}$ (20). After 10 min preincubation, cleavage of the DNA was initiated by the addition of 5 ml of a 12 mM freshly prepared solution of $KHSO_5$ (available as triple salt: $2KHSO_5,KHSO_4,K_2SO_4$; Curox®, a gift from Interox) in 100 mM NaCl, 15 mM arseniate pH 7.2 buffer. After 10 min of cleavage reaction, 200 μl of 1 M HEPES pH 8 buffer were added as stopping reagent (20). Residual starting metalloporphyrin and related residues produced by oxidation, as well as free manganese cations eventually released were eliminated by treatment of the solution with an anionic-exchange resin (Amberlyst® -15-wet from Janssen). Then the sample was lyophilized and purified by a cold water/ethanol (1/3) precipitation. The residue was redissolved in 2 ml of D_2O solution of 0.2 M Tris,HCl pH 7.2. Just before NMR analysis, pH was adjusted to 7.5 with 5M NaOH and the sample was filtered.

DNase I cleavage of DNA

2300 units/ml stock solution of DNase I (from bovine pancreas, Sigma type IV) was prepared in 150 mM NaCl water solution. 1 μl of this solution was added to 10 ml of 3.17 mM DNA solution containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM CH_3COONa adjusted to pH 6.5 with CH_3COOH. After incubation for 1 h at 37°C, the solution was lyophilized and the residue dissolved in 2 ml of D_2O. Before NMR analysis, pH was adjusted to 7.5 with 5M NaOH and the sample was filtered.

DNase II cleavage of DNA

2770 units/ml stock solution of DNase II (from porcine spleen, Sigma type IV) was prepared in bidistilled water. 100 μl of this solution were added to 250 ml of 3.17 mM DNA solution containing 4.8 mM $MgSO_4$, 0.1 M CH_3COONa buffer adjusted to pH 4.6 with CH_3COOH. After incubation for 1 h at 27°C, the solution was lyophilized and the residue dissolved in 3 ml of D_2O. Before NMR analysis, pH was adjusted to 7.5 with 5 M NaOH and the sample was filtered.

Phosphatase treatment of cleavage products

After DNA cleavage with the chemical or natural nucleases, the lyophilized residue was incubated for 1 h (Figure 3B) or 24 h (Figure 3C) at 37°C with 100 units of alkaline phosphatase (from bovine intestinal mucosa, Sigma type VII-NT) in 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 50 mM Tris pH 8.0 buffer. The solution was lyophilized and the residue dissolved in 2 ml of D_2O. Before NMR analysis, pH was adjusted to 7.5 with 10 M HCl and the sample was filtered.

pH dependence of the ^{31}P NMR signal of cleavage products

Immediatly before NMR analysis, some samples were adjusted to pH 5.7 with 10 M HCl and filtered.

^{31}P NMR measurements

^{31}P NMR spectra were obtained in the pulse Fourier transform mode with a Brucker AC 200 spectrometer operating at 81 MHz equipped with a Aspect 3000 computer. The spectrometer was field-frequency locked on the deuterium resonance of deuterium oxide used as a solvent. Spectra were broadband ^1H decoupled. The free induction decays were acquired on 8 K with a sweep width of 4000 Hz. They were Fourier transformed on 16 K after exponential multiplication followed by line broadening of 2 Hz. Other parameters were as follows: 1 sec cycle time, 45 ° pulse length, 20000–40000 scans *per* sample. No pulse delay was

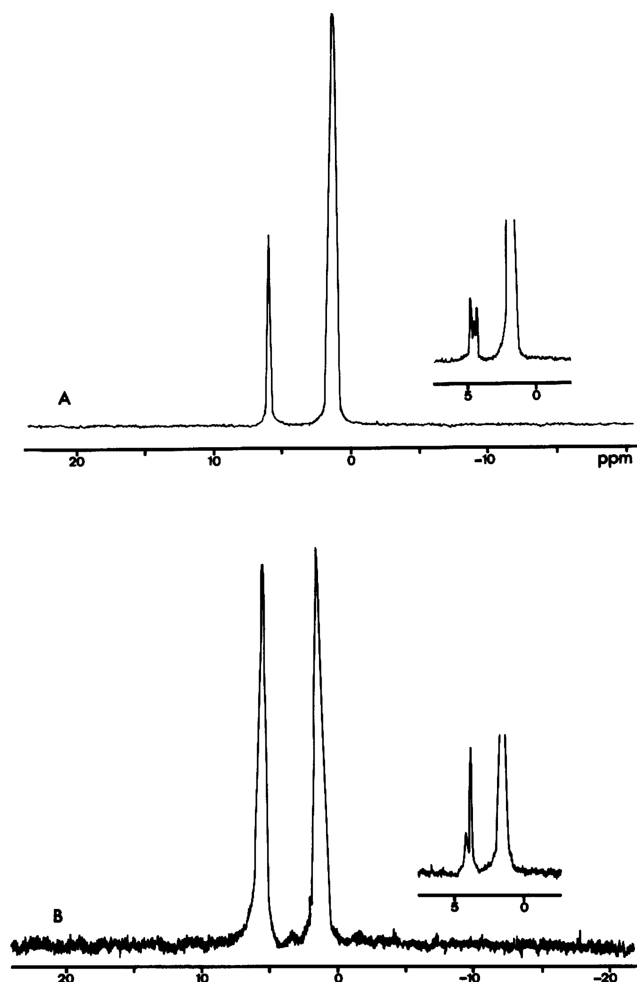


Figure 1. ^{31}P NMR pattern of CT DNA after cleavage by DNase I (A) or DNase II (B). See Figure 2A for the ^{31}P NMR spectrum of sonicated CT DNA before hydrolysis (all experimental details are presented in Materials and Methods). Insets of (A) and (B): upfield shift of monophosphate resonances at pH 5.7.

used. Chemical shifts are referred to the position of resonance of a 85% phosphoric acid solution in D_2O taken as an external standard. Positive chemical shifts are downfield from phosphoric acid. Samples (2–3 ml) were in 10 mm (o.d.) analytical tubes maintained at 298 K during analysis. D_2O (99.8%) from CEA (Gif-sur-Yvette, France) was used as solvent.

RESULTS

Characterization by ^{31}P NMR of terminal phosphates located at the 3′ or 5′ end of calf thymus DNA has been obtained after cleavage of DNA through enzymatic (DNase II or I, respectively) or chemical (Mn-TMPyP/$KHSO_5$) degradation.

Figure 1 compares the ^{31}P spectra of CT DNA after nucleolytic treatment either with DNase I or DNase II. Under both conditions the residual phosphodiesters still present in the fragments appeared as a broad signal at 1.5 ppm (linewidth at half-peak height = 60 Hz), similar in linewidth and chemical shift to that of sonicated CT DNA. As previously noted (3), a sufficiently low DNA molecular weight (obtained by sonication from native DNA) was necessary to provide well resolved ^{31}P spectra (for the use of sonicated CT DNA in oxidative cleavage experiments, see reference 29).

The terminal monophosphates gave signals downfield from the phosphodiester signals. The endonuclease DNase I attacks each strand of DNA independently and the sites of cleavage are distributed in a statistically random fashion; the terminal monophosphate is attached at a 5′ position giving a symmetric and sharp resonance peak at 6.0 ppm. The endonuclease II hydrolyses DNA, forming 3′ terminal oligonucleotides; the corresponding resonance was observed at 5.5 ppm with an unresolved downfield shoulder which might correspond to some 5′ monophosphates due to a residual DNase I activity of DNase II. Assignment of the 6.0 and 5.5 ppm resonances to 5′ and 3′ terminal phosphates, respectively, are in agreement with (i) the observation of Cozzone and Jardetzki (4) for 5′dAMP and 3′dAMP which shows that the 5′ phosphate is shifted 0.4 ppm downfield with respect to the 3′ phosphate (Table 1) and (ii) previous studies on oligodeoxyribonucleotides containing terminal phosphates (5) (Table 1). The appearance of terminal phosphates downfield from the backbone ^{31}P resonances has also been observed previously in transfer (6) and ribosomal (7) RNA (Table 1).

After the chemical cleavage of CT DNA by Mn-TMPyP/$KHSO_5$, the ^{31}P NMR spectrum exhibited a more complex and heterogeneous signal (Figure 2). One rather broad

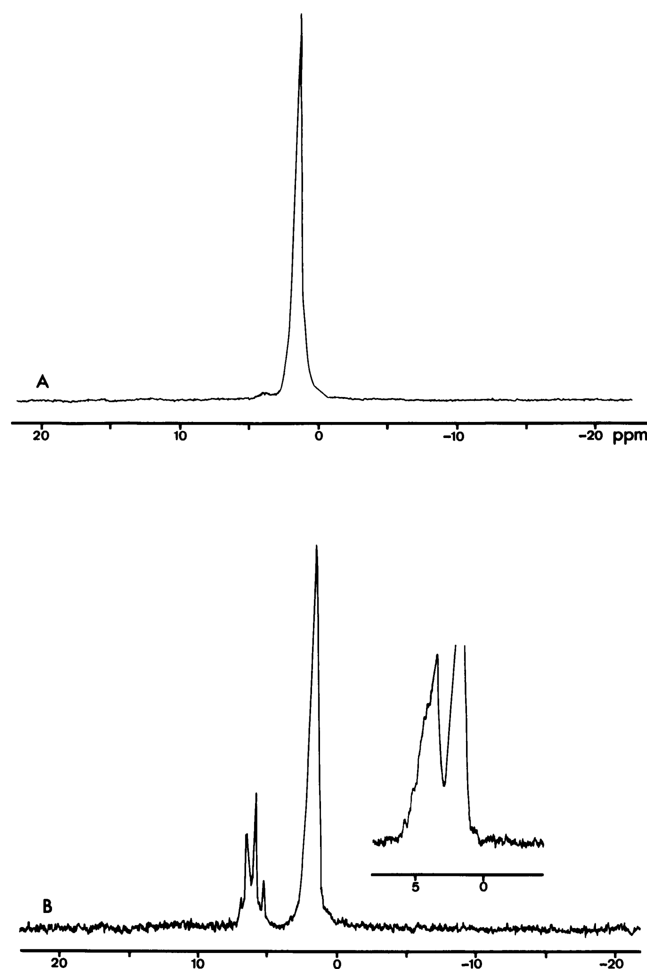


Figure 2. Sonicated CT DNA before (A) and after (B) chemical cleavage by Mn-TMPyP/$KHSO_5$. (inset of (B): upfield shift of monophosphate resonances at pH 5.7).

Table 1. ^{31}P NMR chemical shifts assigned to the terminal 5′ and 3′ monophosphates and phosphodiesters.

Substrate	5′-mono-phosph.	3′-mono-phosph.	Phospho-diester	t°C	pH	Ref.
CT DNA sonicated	–	–	1.5	25	7.5	this work
CT DNA + DNase I	6.0*	–	1.5	25	7.5	id.
CT DNA + DNase II	–	5.5	1.5	25	7.5	id
CT DNA + MnTMPyP/$KHSO_5$**	6.4	5.9	1.9	25	7.5	id.
5'dAMP, 3'dAMP, dApdA	3.8	3.4	−1.1	17	7.5	4
5'AMP, 3'AMP, ApA	3.7	4.1	−0.6	17	7.5	4
oligodeoxyribonucleotides	1.8	1.0	−0.7	0	7	5
rRNA	2.3	2.6	−1.0	30	7	7
$tRNA^{Phe}$	2.7–3.0		−1.0	35	7	6
$tRNA^{Phe}$	3.0	–	−1.5	34	7	1a***

*All chemical shifts (δ ppm) have been taken positively downfield from 85% H_3PO_4 and corrected if necessary to reflect this standard.
**Two minor peaks at 5.2 and 7.0 ppm.
***Ref. 1a, p. 268 (High Resolution ^{31}P-NMR Spectroscopy of Transfer Ribonucleic Acid, D. G. Gorenstein pp. 265–97).

resonance appeared at 1.9 ppm (between 1.7 and 2 ppm) and, downfield, two main resonances at 6.4 and 5.9 ppm with two minor peaks at 5.2 and 7.0 ppm were also present. The first broad signal at 1.9 ppm is unsensitive to pH variations and can be attributed to phosphodiester resonance; the small downfield shift ($\leq$0.5 ppm) observed for this signal compared to the corresponding signal observed during enzymatic cleavage (Figure 1) could be due to the presence of residual metallic residues (starting metalloporphyrin, some metallated degradation products or free manganese cations) as observed for other metallic species (30).

The broad pattern observed between 5 and 7 ppm included four main peaks at 5.2, 5.9, 6.4 and 7.0 ppm. In our experimental conditions, these signals are quantitatively equivalent to that of phosphodiesters (integration of the corresponding areas = 1/1, indicating that fragment sizes statistically correspond to trinucleotides bearing two monophosphate termini). These relative intensities remained nearly constant in all chemical cleavage experiments. We forced the DNA cleavage in order to obtain peaks of high intensities in this part of the spectrum.

These signals can be attributed to monophosphates for different reasons. They shift upfield when the solution pH decreased from 7.5 to 5.7 giving broad resonances centered around 3.4 ppm. Similar behavior has been noticed for monophosphates released by action of DNase I or II (see insets in Figure 1). After treatment by alkaline phosphatase, which is able to catalyze the removal of 5′ and 3′ phosphate residues from DNA (single or double stranded), these signals were completely converted into a sharp single peak at 5.1 ppm characteristic of inorganic phosphate (Figure 3). Overloading experiments confirmed this attribution as well as studies on variations of the chemical shift as a function of pH (data not shown).

Thus the two main peaks at 6.4 and 5.9 ppm can be attributed to, respectively, the terminal 5′ and 3′ phosphates, keeping in mind the downfield shift ($\sim$0.4 ppm) induced by paramagnetic manganese residues. The attributions were confirmed by overloading experiments with oligonucleotides obtained after DNA cleavage with DNase I and II. The two other minor signals at 5.2 and 7.0 ppm are presently unidentified.

During the alkaline phosphatase treatment, two transient residual small signals at 6.4 and 7.0 ppm (Figure 3B) could correspond to monophosphate substrates having a smaller affinity or reactivity towards alkaline phosphatase than the 3′ and 5′ monophosphate termini of DNA. For example, we can exclude the presence of a phosphoglycolate signal, a cleavage fragment possibly generated by chemical nucleases (8, 31), since the addition of the reference compound gave a peak at 5.8 ppm.

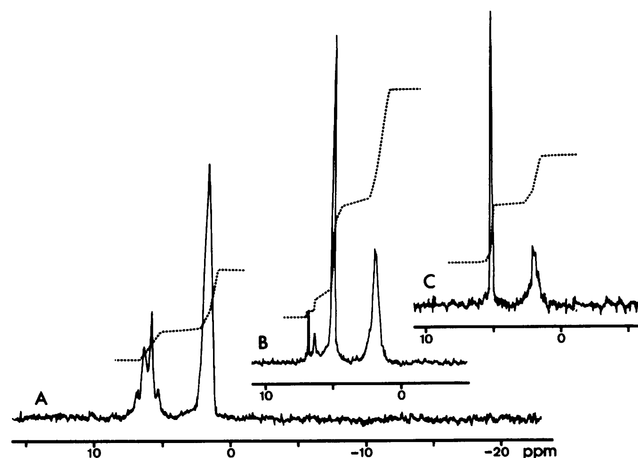
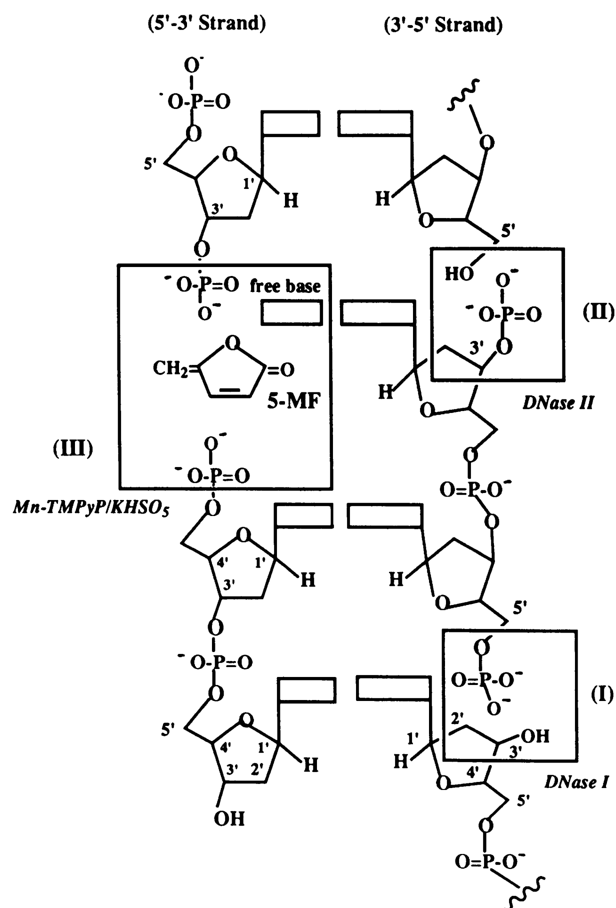


Figure 3. Sonicated CT DNA after chemical cleavage by Mn-TMPyP/$KHSO_5$ (A), and after treatment by alkaline phosphatase for 1 h (B) and 24 h (C).

DISCUSSION

Conformational studies of DNA have been extensively documented using ^{31}P NMR of the phosphodiester bonds because of their sensitivity to torsional angles and to physico-chemical environment (temperature, presence of metal ions, etc.) (1, 2). But more surprisingly, only a limited attention has been paid to the phosphorus magnetic resonance of terminal phosphates.

This study concerns the elucidation of the molecular mechanism of the oxidative cleavage of DNA by the chemical system Mn-TMPyP/$KHSO_5$. We have shown how ^{31}P NMR can support the DNA cleavage mechanism previously proposed on the basis of HPLC analyses of sugar degradation products (23,24). DNA breaks occur through hydroxylation at carbon-1′ (major pathway, Scheme 1, see ref. 23 and 24)) or at carbon-5′ (minor pathway, ref. 24) followed by two β-elimination steps leaving in both cases one phosphate monoester at both sides of the cleavage site.



Scheme I. DNA damages induced by DNase I (I) or DNase II (II) and postulated major cleavage site after the oxidative attack of the Mn-TMPyP/$KHSO_5$ system (III).

As shown above, the ^{31}P NMR resonances corresponding to these terminal 3′ and 5′ phosphates have been identified. However on the basis of the phosphorus NMR spectra, the DNA cleavage by the managanese complex is not as clean as an endonuclease, since there is two other minor unidentified ^{31}P NMR resonances in the monophosphate ester region, suggesting that at least two other monophosphate derivatives exist and suggest other minor pathways.

Considering the clear differences between the ^{31}P NMR pattern of DNA after cleavage by three different nucleases (DNase I, DNase II and Mn-TMPyP/$KHSO_5$), we believe that such NMR approach can constitute a relative simple way to get an insight into the mechanism of cleavage of nucleic acids by drugs known to cleave DNA (8) or for recently developed chemical nucleases (9).

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