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Mechanism of DNA cleavage by cationic manganese porphyrins: hydroxylations at the 1′-carbon and 5′-carbon atoms of deoxyriboses as initial damages

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ABSTRACT

Cationic manganese-porphyrin complexes, free or targetted with an intercalating agent, are able to cleave DNA using oxygen atom donors like potassium monopersulfate or magnesium monoperphthalate as coreactants. Detailed studies of the cleavage of calf thymus DNA, before and after a heating step, show that free bases and 5-methylene-2-furanone are the main reaction products, indicating that hydroxylation at the 1′-carbon atom is the main target of these chemical agents. These data confirm that metalloporphyrin derivatives interact with the minor groove of double-stranded DNA. Hydroxylation of one of the two C-H bonds at position-5′ is another initial DNA damage, characterized by the formation of furfural as sugar degradation product. Besides these two main initial damage sites, a low contribution of a hydroxylation reaction at C4′ can not be definitively discounted, while an hydroperoxidation route at C4′ can be excluded.

INTRODUCTION

The discovery of efficient chemical nucleases (1) and the understanding of the mechanism of action of bleomycin (BLM), an antitumoral antibiotic (2), have been largely developed over the last decade. 'Activated bleomycin', the result of the interaction of BLM with three cofactors (an iron salt, molecular oxygen and two electrons), is able to cleave DNA via the abstraction of the hydrogen atom at the 4′-position of deoxyribose units (3–7). The bleomycin chemistry provided the guidelines for the synthesis of other DNA cleavers based on the attachment of a chelating agent to a recognition moiety able to strongly interact with DNA. The linkage of iron-EDTA to an intercalating agent, an oligonucleotide or a protein, is now the classical example (8–10). Metalloporphyrins have been also used as DNA cleavers. Most of the results were obtained with the iron (Fe-TMPyP) or the manganese (Mn-TMPyP) complexes of *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (11–16), a cationic ligand having a DNA affinity modulated by the central metal atom (17–19). When linked to an intercalating agent, iron or manganese porphyrins can be considered as good models of bleomycin (20–24). These hybrid molecules 'metalloporphyrin-intercalator' have the expected properties of a 'bleomycin-like' molecule: (i) a strong DNA affinity (K_{aff} = 10^8–10^9 M^{-1}), (ii) a good nuclease activity *in vitro* and (iii) a cytotoxicity on whole cells similar to that of bleomycin (22–24). Preliminary studies on the mechanism of DNA cleavage by activated manganese porphyrins indicate that single-stranded polynucleotides are attacked at the anomeric position of deoxyriboses (25). The sugar degradation product resulting from this hydroxylation at C1′ is 5-methylene-2-furanone, 5-MF, as previously observed in DNA breaks mediated by copper-phenanthroline (26, 27).

In the present article, we report evidence that both manganese porphyrin derivatives, Mn-TMPyP and Mn-P-11-Ell (the bleomycin model, see structure on Figure 1), are able to hydroxylate C-H bonds located inside of the minor groove of DNA at C1′ or at the entrance of this groove at C5′. The corresponding sugar degradation product of this latter hydroxylation reaction is furfural (FUR).

EXPERIMENTAL PROCEDURES

Materials

Magnesium monoperphthalate (MMPP), furfural and malonaldehyde bis(dimethylacetal) (MDA) were from Aldrich. Calf thymus DNA (CT DNA), adenine (A), thymine (T), guanine (G), cytosine (C), β-mercaptoethanol were from Sigma. Potassium monopersulfate ($KHSO_5$, Curox®) was a gift of Interox. The two metalloporphyrin derivatives (13, 23) and 5-methylene-2-furanone (28) have been synthesized as described. All the solutions were prepared with doubly distilled water or milliQ water. Bleomycin was a gift of Roger Bellon, France.

General protocol for calf thymus DNA cleavage by metalloporphyrins/oxygen donor systems

DNA concentration (in nucleotides) was controlled by absorbance measurements at 260 nm taking a molar extinction coefficient of 6600 $M^{-1}cm^{-1}$. In a screw cap tube, the reaction medium consisted of 11.5 μL of CT DNA diluted in water (700 μM

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nucleotides), 8.5 μL of 200 mM phosphate buffer pH 8 (34 mM), 5 μL 1M NaCl (100 mM), 12.5 μL of 140 μM metalloporphyrin (35 μM) and 12.5 μL 8 mM oxygen atom donor (2 mM). The final volume was 50 μL; concentrations in parentheses correspond to final concentrations. Before addition of the oxygen atom donor that initiates the cleaving reaction, the constituents were allowed to preincubate at ambient temperature for 15 min. At that time, interaction equilibrium of metalloporphyrin and DNA was reached. The reaction was stopped with 5 μL of 1M Hepes-NaOH buffer pH 8 after 3 minutes (13). The reaction medium was then heated for 15 additional minutes at 90°C and chilled in liquid nitrogen to stop the heated reaction. Injection of aliquots (10, 25 or 50 μL) of reaction medium into chromatographic system were done either immediately after Hepes buffer addition (ambient temperature monitoring) or after the heating and thawing steps.

Analysis of DNA cleavage products

Methodology. HPLC analysis of products formed by reaction of 'activated-metalloporphyrin' with CT DNA were done on reverse phase Interchrom phenyl semi-preparative 10 μ column eluted with 5/95, methanol/5 mM ammonium acetate buffer pH 4.5 (flow rate 1.5 mL/min.). All products were detected at 254 mn; their retention times were: Rt = 7 min (T); 11 min (C); 14 min (5-MF, FUR); 19 min (A). These latter compounds, 5-MF and FUR, could be separated on an Interchrom nucleosil 5 μ C_{18} column eluted with 5/95, acetonitrile/0.1 M triethylammonium acetate pH 6.5 (0.5 ml/min); Rt = 18 min for FUR and 20 min for 5-MF. Quantification of the reaction products (bases, 5-MF) was done by comparison of peak areas *versus* standard injections of authentic samples at different concentrations. Experiments were done in triplicate.

Kinetic study of the products release at ambient temperature. Standard conditions for DNA cleaving reaction were the same as described above except that the reaction was followed over 10 min to measure the increase of bases *versus* time at 20°C.

Kinetic study of the products release at 90°C. After 3 min of reaction at 20°C, appearance of 5-MF and increase of base release were monitored at various times of incubation at 90°C. This was done in the case of Mn-TMPyP associated to MMPP or $KHSO_5$.

Stability of nucleobases in the presence of the catalytic system. See general protocol: 700 μM DNA was replaced by 70 μM of base (A, T, G or C); concentrations of metalloporphyrins were 3 μM (Mn-TMPyP) or 1.6 μM (Mn-P-11-Ell).

Degradation of 5-MF at 90°C. The kinetics of degradation of 5 MF in the reaction conditions was studied as follows. A solution containing 60 μL of 1 M NaCl, 102 μL of 200 mM phosphate buffer pH 8,150 μL of 140 μM Mn-TMPyP and 150 μL of 8 mM oxygen donor ($KHSO_5$ or MMPP prepared just before use) was incubated at room temperature for 3 min. Then were added 60 μL of 1 M Hepes/NaOH buffer pH 8, 18 μL of 1 mM adenine (internal reference) and 120 μL of 415 μM 5-MF (final concentration 79 μM). Aliquots (60 μL) were heated at 90°C in screw cap tubes and the reaction was stopped at various times in liquid nitrogen. 5-MF was assayed as indicated above.

Cleavage of CT-DNA by bleomycin/Mn-TMPyP. To 59.5 μL of a solution containing 57 mM phosphate buffer pH 8, 168 mM NaCl, 1.13 mM DNA and 1.68 mM bleomycin were added 10 μL of 10 mM $Fe(NH_4)_2(SO_4)_2$ to initiate DNA cleavage by iron bleomycin. The reaction is allowed to stand 30 min at 0°C, then 25 μL of 140 μM Mn-TMPyP (or 25 μL H_2O) were added to the reaction mixture. After a 15 min preincubation time, 25 μL of a 8 mM $KHSO_5$ or MMPP solution initiated the second DNA cleaving system. Reaction was allowed to take place for 5 min at 20°C before addition of 10 μL of 1.0 M Hepes-NaOH buffer pH 8. In control experiments, an equal volume of water replaced the omitted reagent.

Analysis of base propenals. The base propenals were quantified by TBA/malondialdehyde reaction. A solution of 0.6% TBA (0.9 mL) was added to 0.1 mL of sample (DNA cleavage reaction in the presence of either BLM or porphyrin systems, or both systems, see above). Then the solution was heated 20 min at 80°C. After cooling at room temperature, absorbance at 532 nm was measured and malondialdehyde was quantified against authentic standards (29).

Cleavage products were analyzed by thin layer chromatography (30): 50 μL of reaction mixture were applied on silica plate and eluted with ethyl acetate/ethanol/H_2O/acetic acid (62.5/27/9/1.5; v/v/v/v). Bases were detected by exposure to UV light (254 nm)

Table 1. Release of bases and 5-MF after 3 min of reaction at ambient temperature followed by 15 min heating at 90°C. Columns are micromolar concentrations of products.

	Before heating				After heating		
	BASES[a]						
Reagent	A[b]	C[b]	T[b]	Total bases[c]	5-MF[b]	base increase[d]	ratio 5-MF /total bases
Mn-TMPyP							
+ $KHSO_5$	60 ± 5[e]	36 ± 6	60 ± 5	156	98	+ 10%	0.63
+ MMPP	25 ± 2	30 ± 3	21 ± 1	76	75	+ 20%	1.0
Mn-P-11-Ell							
+ $KHSO_5$	17 ± 2	28 ± 2	23 ± 2	68	26	nd[f]	0.38
+ MMPP	6 ± 0.5	6 ± 0.5	6 ± 0.5	18	16	nd	0.88

[a]G was never detected in the experimental conditions (see Results section).
[b]Corrected values for bases and 5-MF release according to their partial degradation during their formation at ambient temperature (bases) or when heating (5-MF) (see Results section).
[c]Total bases = A + C + T in μM.
[d]Significant increase of free bases release was determined during the heating step.
[e]Mean value ± standard deviation (three determinations).
[f]nd: not determined.

and base propenals were identified by spraying the plate with 0.6% TBA and heating for 1 hour at 80°C (Rf were: A = 0.45; C = 0.20; G = 0.35; T = 0.80, base propenals respectively: 1.0; 0.80; 0.65 and 0.55).

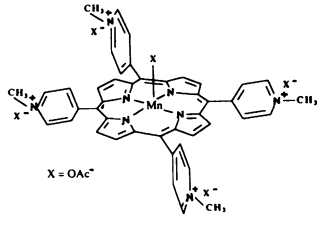
Bases and base propenals released were also analyzed by HPLC on a 10 μ Waters C_{18} column with a linear gradient of 5 mM ammonium acetate pH 4.3/methanol 0−100% in 20 min at a flow rate of 1.5 mL/min. Bases and base propenals were detected at 254 and 313 nm; Rt: G = 2.8 min; T = 3.0 min; C = 4.4 min; A = 5.0 min; base propenals respectively: 3.8; 5.6; 6.6 and 11.6 min. Bases were identified against authentic standards; base propenals were identified by the disappearance of their signals when the bleomycin digest was realized in the presence of 8 mM β-mercaptoethanol (30) or after heating the reacting medium.

Analysis of sugar derivatives. FUR and 5-MF have been characterized as previously described (25, 31).

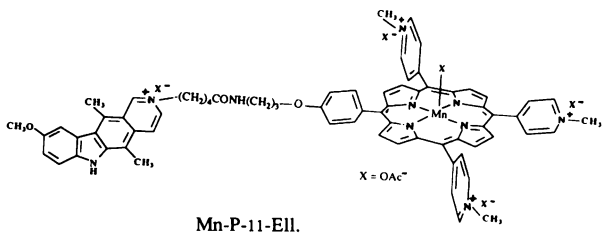
RESULTS

Nucleic acid bases released in CT DNA cleavage mediated by manganese porphyrins

HPLC analyses indicated that calf thymus DNA (61% of AT and 39% of CG base-pairs) damage leads to the release of free nucleobases: after 3 min in experiments with $KHSO_5$ and 1 min in experiments with MMPP a plateau was reached for the three observed nucleobases (A, C and T; the case of G will be discussed below). The quantitative results of base release at 3 min of reaction are presented in Table 1 (values have been corrected according to their partial degradation in the medium, see the next paragraph). Adenine, cytosine and thymine were released in rather similar proportions. Guanine was never detected; this result was not surprising in the case of the $KHSO_5$/Mn-TMPyP cleaving system due to the fast degradation of free guanine by such a strong oxidizing reagent (see below). Guanine could have been released and rapidly oxidized in the reaction medium. However this cannot explain completely the lack of guanine in the experiments with the three other Mn-porphyrin nucleases since controls demonstrated that guanine was not totally oxidized by these systems. So, in these cases, if guanine is effectively released during the reaction it is probably in very small amount. The total yield of observed base release was maximum for $KHSO_5$/Mn-TMPyP (see Table 1).



Mn-TMPyP



Mn-P-11-Ell.

Figure 1. Structures of porphyrin derivatives Mn-TMPyP and Mn-P-11-Ell.

In the presence of the whole catalytic system, the nucleobases were partially (A, T, C) or heavily (G) degraded. As bases were liberated only in the early stage of the cleavage reaction (< 1 min, MMPP, or < 3 min, $KHSO_5$) because of the fast inactivation of the metalloporphyrin by self-degradation (bleaching effect) (15, 24), their degradation can only happen during this initial step. In order to determine an upper limit to the amount of base release, we studied their stability in the conditions of cleavage experiments without DNA but in the presence of the calculated amount of free metalloporphyrin (according to recently determined binding constants values, references 23−24), respectively 3 and 1.6 μM for Mn-TMPyP and Mn-P-11-Ell. The major part of the initial amount of the metalloporphyrin was bound to DNA.

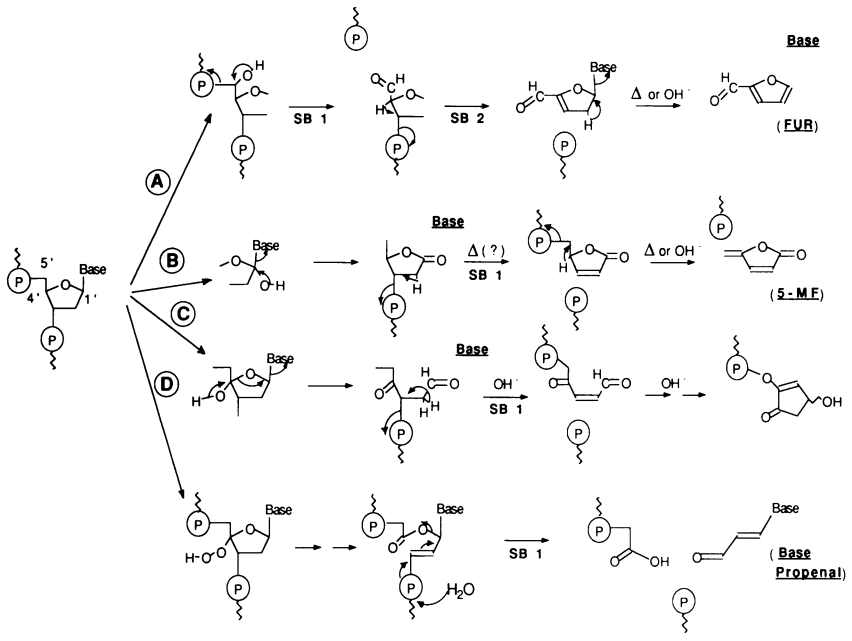
With Mn-TMPyP, the observed percentages of base degradation were: A, 40%; T, 60%; C, 50%; G, 100% (for $KHSO_5$) and A, 5%; T, 15%; C, 25%; G, 40% (for MMPP). With the bleomycin model, Mn- P-11-Ell, base degradations in the presence of $KHSO_5$ were as follows: A, 20%; T, 40%; C, 50%; G, 60%; for MMPP, no degradation occurred for any bases. The values for base release in Table 1 have been corrected according to these data. The corrected total value represents only a lower value since there is still a doubt about the release of G (which was never detected).

Considering the corrected results for total bases released (in fact, A + T + C; see Table 1), as well as the corresponding values observed for corrected 5-MF (see below), it seems that, except in the case of Mn-P-11-Ell/$KHSO_5$, the free bases and the sugar residue were formed in approximately similar amounts. A ratio 'corrected 5-MF/corrected total of released bases' equal to 1.0 is expected for a specific hydroxylation at C1′ (5-MF is a marker of hydroxylation at C′1). So, except for Mn-P-11-Ell/$KHSO_5$, these data suggest that carbon-1′ was the main target for the hydroxylation of DNA by the metalloporphyrin catalytic systems. At least for Mn-P-11-Ell/$KHSO_5$, another (other) mechanism(s) leading to a release of free bases without the formation of 5-MF should be involved.

Quantification of 5-MF

Hydroxylation of DNA by Mn-TMPyP/$KHSO_5$ occurs mainly at the carbon-1′ of deoxyribose and, after a heating step, induces the release of 5-MF as oxidative degradation product of the sugar moieties (Scheme I, pathway B). Since 5-MF is heat-sensitive, we performed a kinetic study of its appearance in the course of the thermal step at 90°C following the DNA cleavage reaction mediated by the metalloporphyrin and the complete degradation of the oxygen atom donor in excess ($KHSO_5$ or MMPP). The maximum of 5-MF was observed after heating 10 min the reaction medium, then it decreased to reach an equilibrium value, indicating a transformation of 5-MF in the experimental conditions (Figure 2A and Table 1).

In a control reaction, without DNA, and starting with an initial 5-MF concentration of 79 μM, we observed the partial disappearance of 5-MF reaching a plateau (30 μM at 45 min, Figure 2B). Kinetics were similar whatever the oxygen atom donor used ($KHSO_5$ or MMPP) indicating that these oxidants



Scheme I. Different pathways for different oxidative attacks on C-H bonds of DNA deoxyriboses by a chemical nuclease having an affinity for the minor groove (the C-H bond at C3′ is only accessible from the major groove). SB 1 and SB 2 = first and second strand breaks. P = terminal phosphate residue.

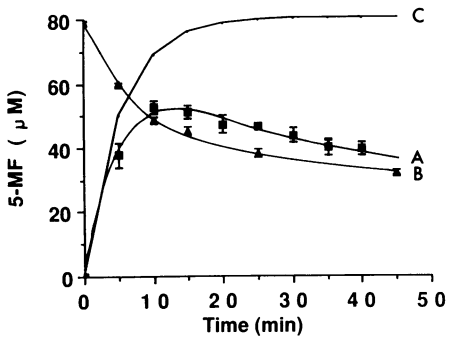


Figure 2. (A) Formation and evolution of 5-MF during the heating step following CT DNA cleavage by Mn-TMPyP/$KHSO_5$ according to the general protocol; (B) Kinetic evolution of a known amount of reference 5-MF (79 μM) in the conditions of DNA cleavage; (C) Theoretical cumulative release of 5-MF during the thermal step.

were completely destroyed by the Hepes buffer (stopping reagent) and did not play a role in the 5-MF evolution. A classical kinetic analysis of the 5-MF disappearance shows a good linearization (R^2 = 0.99) for $Ln([5\text{-MF}]-[5\text{-MF}]_\infty)$ = f(t) with [5-MF] and $[5\text{-MF}]_\infty$ = concentrations of 5-MF respectively at time t and t_∞ (t_∞ = time necessary to reach the equilibrium = ~60 min) (32). This equilibrium should correspond either to the hydration of the C4–C5 double bond or to the opening of the lactone ring of 5-MF (33) (details on kinetics are available on direct request to the authors). So all the corrected values for 5-MF in Table 1 have been calculated from $[5\text{-MF}]_{max} \times 1.5$ according to kinetic parameters of the reaction ($[5\text{-MF}]_{max}$ = maximum of observed 5-MF concentration).

Furfural as marker of DNA hydroxylation at 5′-carbon of deoxyribose

Recently we have shown that furfural, FUR, is the oxidized sugar residue resulting for an hydroxylation at carbon 5′ of deoxyribose (31). This compound seems to be only a minor product of the chemical cleavage of CT DNA. Due to its relative instability when heated and the low amount released during the heating step, the molar ratio of FUR with respect to 5-MF can be only estimated to 15 ± 5%. Existence of this minor mechanism was further supported by the observed increase of free bases during the heating step (+ 10% and + 20% respectively after the 15 min thermal step in the experiments with $KHSO_5$/Mn-TMPyP and MMPP/Mn-TMPyP). As described in Scheme I, when free bases are released in the course of the cleavage reaction (i.e. before the thermal step) in the C1′-hydroxylation route (pathway B), another amount of free bases should be released after heating in the C5′-hydroxylation route (pathway A). In both cases sugar residues (5-MF and FUR) were only liberated during the heating step.

Possible oxidative attack at 4′-carbon of deoxyribose by Mn-porphyrin nucleases

Refering to the mechanism of DNA cleavage by bleomycin (5 and references therein) (pathway C and D in Scheme I), we have investigated the possibility of a hydrogen abstraction at C4′. Since DNA digestions by activated manganese porphyrins are conducted in air, the oxygen-dependent route (pathway D) might give rise to base-propenal residues as sugar degradation products. So MDA assays and determinations by TLC and HPLC of base-propenals were performed after DNA digestion by (i) Fe(II)-BLM alone, (ii) Fe(II)-BLM and activated Mn-porphyrin (in order to check the stability of base-propenals in the presence of the Mn-porphyrin nuclease), and (iii) activated Mn-porphyrin alone. No

base propenals were detected during the oxidative cleavage of DNA by the porphyrin systems; we checked that base propenals produced by BLM were nearly stable in the presence of activated Mn-porphyrins. Thus the possibility of route D can be discounted. Concerning a possible non-oxygen dependent 4′-hydroxylation route (Scheme I, pathway C), it must be noted that such sugar degradation process does not release any detectable sugar marker in solution, even after an alkaline treatment. Up-to-now, we cannot definitively rule out a minor contribution of such a mechanism in DNA cleavage by activated manganese porphyrins.

DISCUSSION

Little is known on the molecular aspects of DNA cleavage by activated manganese or iron porphyrin complexes. These 'chemical nucleases' are unable to intercalate between DNA base-pairs and are supposed to bind to the outside of DNA (18). From gel-sequencing studies, it is clear that the preferred cleavage site is an AT triplet (34). Theoretical investigations have proposed that, even for the non-metalated porphyrin, H_2TMPyP, there is a strong interaction of this ligand within the minor groove in AT-rich regions, besides the generally accepted intercalation between GC base-pairs (35). Preliminary work (25) on poly dA has shown that sugar units of this polynucleotide can be hydroxylated at the anomeric carbon atom, C1′, giving rise to the liberation of 5-methylene-2-furanone after a thermal step (Scheme 1, pathway B). Such attack site has been reported as being the major target of bis(1,10-phenanthroline)copper in DNA cleavage (26). Presently we analyze the reaction products of double stranded DNA cleaved with cationic manganese porphyrins in order to check if C1′ of deoxyribose is the only, or the main, hydroxylation site (if C1′ is the only target the ratio 5-MF *versus* free bases should be equal to 1.0). We have to keep in mind that the C1′-H bond is more inside of the minor groove compared to the C-H bond at C4′ or the C-H_S bond at C5′ (H_S stands for the hydrogen atom which is oriented toward the minor groove, H_R being the one oriented toward the major groove). The abstraction of a hydrogen atom at C4′ and C5′ has been largely documented in the case of bleomycin (4, 5) and neocarzinostatin (36 and 37). Even if we take in consideration the limitations concerning the G release (see above), it is clear from the ratio detected bases/formed 5-MF (see Table 1) that hydroxylation at C1′ is the main damage leading to DNA strand breaks (SB 1 and SB 2 in pathway B, Scheme 1). The first β-elimination in the lactone intermediate liberates a phosphate terminus from the 3′-position and leaves the 5-MF precursor still attached to the 5′-phosphate. The second β-elimination is very slow at room temperature. The 5-MF liberation is only observed after a thermal activation (15 min at 90°C). The hydroxylation at C1′ is performed by both manganese porphyrin derivatives: Mn-TMPyP and the bleomycin model Mn-P-11-Ell.

Besides the hydroxylation at C1′, we looked for other possible sugar hydroxylation sites accessible from the minor groove. The H_S atom at C5′ and the one at C4′ are two potential targets. Hydroxylation at C5′ is signed by two initial strand breaks (SB 1 and SB 2 in pathway A, Scheme I), and by the release of free bases and formation of furfural in the thermal step following the DNA cleavage reaction (31). In all CT DNA cleavage experiments by one of the four Mn-porphyrin nucleases, we found that FUR is formed, as well as a slight increase of free bases is observed after the thermal step, However, the ratio FUR/5-MF is low, 15 ± 5%, suggesting that the hydroxylation at C5′ is a minor pathway in the CT DNA cleavage mediated by activated metalloporphyrins. These results have been obtained in drastic cleavage conditions (i.e. with one metalloporphyrin for twenty nucleotides) in order to facilitate the detection and the identification of sugar degradation products. So, at low metalloporphyrin loading (in experimental conditions corresponding to usual gel-sequencing analyses, one single hit for two or three hundred base pairs), we cannot prejudge the relative importance of these two routes and their responsibilities for DNA strand breaks.

Concerning the hydroxylation of the C-H bond at C4′, we checked that the oxygen-dependent route leading to the generation of base-propenals (pathway D, Scheme I) is not operating in DNA cleavage by manganese porphyrins. The last possible mechanism is the non oxygen dependent hydroxylation occurring at C4′ (pathway C, Scheme I). We are currently working on this hypothesis in order to have an overview as complete as possible on all potential sugar hydroxylation sites which are accessible for a metal-oxo complex, a non-diffusible species, generated within the minor groove of DNA.

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