DNA intercalation and photosensitization by cationic *meso* substituted porphyrins

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ABSTRACT

Several cationic porphyrins are known to bind to DNA by intercalative and outside binding modes. This study identifies the *cis* and *trans* isomers of bis(N-methyl-4-pyridiniumyl)diphenyl porphyrin as DNA intercalators based on evidence from a DNA topoisomerase I assay. Moreover, both isomers are shown to be potent photosensitizers of DNA, inducing multiple S1 nuclease sensitive breaks in the phosphodiester backbone. Porphyrin-induced photodamage in DNA was also shown to be quantitatively dependent upon ionic strength and to inhibit the action of restriction endonucleases. The results indicate that these porphyrins can be useful probes of DNA structure and have potential as DNA-targeted photosensitizers.

INTRODUCTION

Since the original observation(1) and subsequent confirmation(2) that N-methyl pyridyl cationic porphyrins could bind to DNA by intercalative and outside binding modes, a large body of work has evolved describing the details of this complex interaction. Recently, a number of review articles have been published that discuss the status and implications of this work.(3-6)

Our original objective in studying cationic porphyrins was to develop DNA-targeted photosensitizers to study photosensitized-induced damage in DNA. However, the unique and unanticipated high affinity of these cationic porphyrins for DNA diverted our attention to a long term investigation of their binding properties. As a result of our effort and the experimental and theoretical work of a number of other researchers, we now have a reasonable description of the complexes formed between tetra cationic porphyrins and DNA. Moreover, we believe that this knowledge greatly enhances the value of the cationic porphyrins as investigative tools with which to examine the action of photosensitizers on nucleic acid substrates.

Until recently, the work in this field has focused on tetra-cationic porphyrin derivatives as typified by *meso*-tetrakis(N-methyl-4-pyridiniumyl)porphyrin. However, the comprehensive paper by Sari, et al.(7), which examines the effect of the number and position of positive charges on the mode and strength of binding of the porphyrin has stimulated interest in mono, di and tri-cationic derivatives. The *cis* and *trans* bis(N-methyl-4-pyridiniumyl)diphenylporphyrin, in particular, have gained

attention and have raised some interesting questions.(7-9) Sari, et al.(7) have concluded that both the *cis* and *trans* di-cationic porphyrin bind to DNA by intercalation. Gibbs, et al.(9) believe that neither of these dicationic porphyrins are 'particularly good intercalators', and prefer to bind externally to form stacked structures which report on the helical sense of DNA.

An earlier report from our laboratory described the ability of *meso*-tetrakis(4-trimethylaniliniumyl)porphyrin [TMAP], to bind externally in a self-stacked array along the surface of DNA.(10,11) In this case, however, no evidence was found for intercalative binding. Our observation was confirmed by Banville, et al.(12) and 'self-stacked' outside binding was classified as one of the three DNA binding modes identified for cationic porphyrins.(4,6)

Although the difference in the conclusions put forth by Sari, et al.(7) and Gibbs, et al.(9) regarding the intercalative binding of the dicationic porphyrins may simply reflect a difference in experimental conditions, understanding these complex binding modes is critical to understanding the photosensitizing activity of cationic porphyrins on nucleic acid substrates. Toward this goal, the present work reexamines the nature of the binding of tetra, tri, di and monocationic porphyrins to DNA using a topoisomerase I-DNA-unwinding assay(13) and reports on the photosensitizing activity of *cis* and *trans* porphyrin.

MATERIALS AND METHODS

Porphyrins

meso-Tetrakis(N-methyl-4-pyridiniumyl)porphyrin [T4mPyP], *meso*-tris(N-methyl-4-pyridiniumyl)porphyrin [tricationic porphyrin], *cis-meso*-bis(N-methyl-4-pyridiniumyl)diphenyl porphyrin [*cis*-dicationic porphyrin], *trans-meso*-bis(N-methyl-4-dipyridiniumyl)diphenylporphyrin [*trans*-dicationic porphyrin] and *meso*-(N-methyl-4-pyridiniumyl)triphenylporphyrin [monocationic porphyrin] were purchased from Porphyrin Products, Logan, Utah and Midcentury Co., Chicago, IL. Porphyrin structures are shown in figure 1.

DNA

A 4144 bp plasmid(pGS81) was used for this study and was derived from pBR322 in which a 440 base pair fragment containing the *E. coli* origin of replication was substituted for the *Pst*I to *Eco*RI segment of the ampicillin resistance gene (*bla*). It was isolated from *E. coli* HB101 using hydroxyapatite.(14)

Light exposure

The porphyrin was added to plasmid DNA in a 1.5 ml. eppendorf microtube and placed 4 cm from a KenRad F15T8/D 15 watt fluorescent daylight lamp which delivered a light intensity of approximately 0.90 Lux. The photoexposure ranged from 4.0×10^{-5} to 10^{-4} joules/cm² in various experiments.

Agarose gel electrophoresis

After exposure to porphyrin and/or light, DNA was either used directly or first extracted with buffer saturated phenol, to remove the porphyrin, precipitated with isopropanol and run on 1% GTG agarose (FMC) in tris-EDTA, pH 8.0, at 2 volts/cm for 90 min. After electrophoresis, the gel was stained for 60 min. in 1 μ g/ml of ethidium bromide and photographed.

DNA unwinding experiments

Assays were performed in two steps. First, closed supercoiled (form I) pGS81 (0.5 μ g.), was relaxed with wheat germ topoisomerase I (Promega Corp., Madison, WI). This was followed by a second topoisomerase I mediated relaxation in the presence of porphyrin. A second relaxation in the presence of an intercalator results in negatively supercoiled DNA.(13,15) Specifically, topoisomerase I (2 units) was incubated at 37 deg. C for 30' with 0.5 μ g of form I pGS81 DNA first in the absence and then in the presence of porphyrin in 50 μ l containing: 50mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 1mM DTT and 20% glycerol. The topoisomerase I assay was stopped with 5 μ l of 10% SDS, extracted with 50 μ l of buffer saturated phenol and 50 μ l of water. After mixing and centrifugation, the upper aqueous phase was precipitated with isopropanol, dried, dissolved in sample buffer and subjected to agarose gel electrophoresis. After addition of the cationic porphyrin, care was taken to prevent exposure to light.

Figure 1. Structure of DNA interactive porphyrins. A. *meso*-tetrakis(N-methyl-4-pyridinium)porphyrin, T4mPyP; B. *meso*-tris(N-methyl-4-pyridinium)phenyl porphyrin, tricationic porphyrin; C. *trans-meso*-bis(N-methyl-4-pyridinium)diphenyl porphyrin, *trans*-dicationic porphyrin; D. *cis-meso*-bis(N-methyl-4-pyridinium)diphenyl porphyrin, *cis*-dicationic porphyrin.

RESULTS

DNA intercalation by mono, di, tri and tetracationic porphyrins

A convenient way to assess the ability of a DNA ligand to intercalate is to measure the relaxation of ligand induced positively supercoiled DNA using eucaryotic topoisomerase I. An intercalating agent produces negatively supercoiled DNA(13,15) that can be readily detected by agarose gel electrophoresis. T4MPYP has been identified as an intercalator using a topoisomerase I(16,17) confirming our earlier findings using a conventional agarose gel electrophoresis assay in which porphyrin is incorporated into the gel.(2) We have also used the conventional agarose gel electrophoresis method to demonstrate intercalation of the tricationic porphyrin (unpublished observation); however, the fluorescence from the porphyrin interferes to some degree with visualization of DNA in the gel. The *cis* and *trans* dicationic porphyrins cannot be evaluated (data not shown).

It is clear from the results of the topoisomerase I assay shown in Figure 2, that the tetra, tri, and dicationic porphyrins (*cis* and *trans*) bind to DNA by intercalation, ie., all induced negative supercoils. Nearly equivalent concentrations of the tricationic and tetra cationic porphyrin (3 μ M) produces a similar degree of unwinding. Slightly higher concentrations of the dicationic porphyrins are required (5 μ M), but the *cis* and *trans* isomers fully unwind the DNA at similar concentrations as seen in Figure 2b lanes 4–8 and 10–14.

Figure 2. Agarose gel electrophoresis of supercoiled DNA relaxed by topoisomerase I in the presence of cationic *meso* substituted porphyrins. Panel A contains form I and II marker DNA (lane 1) and form I DNA (0.5 μ g) relaxed in the presence of 0, 0.75, 1.5, 3.1, 6.2, 12.5 and 25 μ M of the tricationic and the tetracationic porphyrin, lanes 3–8 and 10–15 respectively. Lane 9 contained 50 μ M tricationic porphyrin. Panel B lanes 1 & 2 are the same as panel A. Lanes 4–8 and 10–14 contain: 0.3, 0.6, 1.25, 2.5, & 5.0 μ M *cis* and *trans* dicationic porphyrin respectively but only 0.2 μ g of DNA was analyzed.

Photosensitized cleavage of DNA by dicationic porphyrins

Photosensitization activity and photophysical properties of many porphyrins have been extensively investigated. Several porphyrins have been shown to mediate DNA cleavage, including T4MPYP.(18,19) Figure 3 demonstrates the results of photo-induced DNA nicking of DNA with both the *cis* and *trans* dicationic porphyrin. A decrease of covalently closed form I DNA occurs within 5 min of exposure to light (lanes 4 & 12) and the form I DNA completely disappears by 30 minutes. Continued illumination of the DNA alters the form II DNA such that it migrates in the gel at a somewhat faster rate. This is seen by comparing the form II species in lanes 10 & 11 and lanes 18 & 19. Small amounts of linear form III DNA begin to appear after approximately 15 minutes but even after 120 min the rate modified from II DNA is still the largest fraction of DNA.

Sensitivity of porphyrin-light treated DNA to S1 nuclease

One possible explanation for the occurrence of a 'rate-modified' form II DNA is that porphyrin-photosensitization induces multiple nicks in the phosphodiester backbone and that this action modifies form II DNA, which preserving its closed circular form. To test this, porphyrin-photosensitized DNA was treated with S1 nuclease. If extensive nicking is present, S1 nuclease should rapidly degrade the DNA. S1 digestion of porphyrin-photosensitized plasmid DNA does indeed result in rapid DNA

Figure 3. Agarose gel electrophoresis of *cis* and *trans* dicationic porphyrin photosensitized plasmid DNA. Plasmid DNA (1.0 μ g.), 20 mM Tris-HCl (pH 8), 0.5mM EDTA and 2 μ M porphyrin were placed in 100 μ l. and exposed to a daylight fluorescent lamp. Ten μ l of each was withdrawn at: 0, 5, 10, 20, 30, 45, 60, and 120 min. (lanes 3–10 and 11–18 *cis* and *trans* dicationic porphyrin respectively). This photoexposure varies from 4.0×10^{-5} joules/cm² at 5 min. to 10^{-4} joules/cm² 120 min. Lane 1 is untreated form I and II DNA and lane 2 contains form III linearized plasmid. Lane 19 contains native plasmid DNA exposed to light in the absence of porphyrin for 30 min.

Figure 4. Agarose gel electrophoresis of native and phototreated plasmid DNA digested with S1 Nuclease. Plasmid DNA (2 μ g) and 2 μ M *cis* dicationic porphyrin was placed in 50 μ l of 50 mM NaAc pH 6 and either kept in the dark or exposed to 7.2×10^{-4} joules/cm² at 30°C. The DNA mixtures were then digested, in the dark, with S1 nuclease at 37°C. Samples of both the dark control (lanes 1–7) and light treated (lanes 8–14) were taken at 0, 10, 20, 30, 45, 60, and 90 min. respectively and analyzed by agarose gel electrophoresis.

degradation (Figure 4, lanes 8–14). After just 10 minutes the modified form II DNA had been almost completely converted to linear molecules some of which migrated as form III and the remainder degraded to a population of short fragments seen as a smear migrating ahead of form III. After 45 min. all of the DNA had been degraded. The lack of discrete bands is indicative of a broad population of low molecular weight fragments. This occurs when there are a large number of different sites at which single-strand breaks occur. In contrast, S1 digestion of native form I plasmid DNA is much slower. After 10 min. nearly all of form I molecules were digested with most of the product as nicked circular form II (lane 2). However, this resulting form II DNA, is much more resistant to further S1 digestion. Whereas the porphyrin-photosensitized plasmid DNA was extensively degraded within 10 minutes, greater than 50% of the form II DNA remains after 90 min.

Heat-denaturation of porphyrin-photosensitized plasmid DNA

Another way to analyze the porphyrin-photosensitized plasmid DNA for multiple nicks is to determine whether thermal stability

Figure 5. Agarose gel electrophoresis of photosensitized and control plasmid DNA exposed to increasing temperatures. Plasmid DNA (1.0 μ g), 20 mM Tris-HCl (pH 8), 0.5mM EDTA and 2 μ M *cis* dicationic porphyrin were placed in 100 μ l and exposed to a daylight lamp for 4.8×10^{-4} joules/cm². As a control, plasmid DNA was linearized with *Pst*I and subjected to the same conditions of buffer and temperature. Twenty μ l aliquots of the light/drug treated (lanes 2–6) and control (lanes 7–11) were placed in separate eppendorf tubes and heated for 10 min. to 30°, 45°, 55°, 65°, and 90°C. respectively and subjected to electrophoresis.

Figure 6. Agarose gel electrophoresis of *Pst*I digested photosensitized plasmid DNA. Plasmid DNA (1.0 μ g) was placed in 100 μ l containing 20 mM Tris-HCl, pH8.0, 0.5 mM EDTA and 2 μ M *cis* dicationic porphyrin and exposed to daylight of 0.90 lux. Samples (0.2 μ g) were withdrawn at 0, 15, 30, 60 & 90 min. and divided equally. One-half was electrophoresed directly (lanes 1–5) and the remainder was digested with 2 units of *Pst*I restriction endonuclease (lanes 6–10) followed by agarose gel electrophoresis. Endonuclease digestion of control DNA in the presence and absence of the *cis* dicationic porphyrin is shown in lanes 11 & 12.

Figure 7. Agarose gel electrophoresis of plasmid DNA phototreated in the presence of porphyrin and mono- and divalent cations. Plasmid DNA (1.0 μ g), 20 mM Tris-HCl (pH 8), 0.5mM EDTA and 2 μ M *cis* dicationic porphyrin were placed in 50 μ l with 0, 2, 10, 50, 100, 200, & 500 mM NaCl (lanes 2–8 respectively) and 0, 1, 5, 10, 25 mM $MgCl_2$ (lanes 9–13 respectively, lane 14 is identical to lane 9). Lanes 1 and 15 contain Form I, II and III marker DNA. The DNA was exposed to a daylight lamp for 4.8×10^{-4} joules/cm² and analyzed by agarose gel electrophoresis.

is decreased. The porphyrin-photosensitized plasmid DNA was subjected to increased temperatures and analyzed by gel electrophoresis. As seen in Figure 5 the porphyrin-photosensitized DNA is more readily heat-denatured than control. The photosensitized modification was somewhat more extensive in this experiment as judged by the presence of linear DNA in addition to modified form II. Approximately one-half of the modified DNA is denatured at 65°. At 90°, the modified DNA is completely denatured resulting in a 'smear' of small fragments. The linearized *EcoRI* control is completely stable at this temperature. (lane 11).

Photosensitized damage to DNA interferes with restriction endonuclease cleavage

Nucleolytic enzymes have been used extensively as tools to probe changes in DNA structure.(20,21) We demonstrate that the *cis* dicationic porphyrin-photosensitized DNA inhibits digestion by *PstI* restriction endonuclease. Lanes 1–5 of Figure 6, show the effects on plasmid DNA after exposure to light (0 to 90 min.) in the presence of 2 μ M *cis*-porphyrin. Clearly, DNA is progressively altered as a function of time. DNA in the dark control is completely digested with *PstI*. The porphyrin-photosensitized DNA becomes resistant to *PstI* digestion as a function of exposure time (lanes 7–10). Other restriction endonucleases including *EcoRI*, *NruI* & *SalI* are similarly affected to differing degrees (data not shown).

Mono and divalent cation counterion effect on the DNA photosensitized damage and intercalation

It has been shown that T4MYP and related porphyrins cause strand breaks in DNA via singlet oxygen based upon evidence from inhibitor studies(18). The effectiveness of these ligands to promote photochemical damage probably depends on a number of factors including their proximity to the target. One condition that may be required for the cationic porphyrin to efficiently promote the DNA photodamage is DNA binding and it is well known that the degree and mode (intercalator vs. outside) of binding is a function of ionic strength.(4,6) Mono and divalent cation counterions are known to attenuate drug binding.(23) As seen in Figure 7, mono and divalent cations decrease photodamage in a concentration dependent manner. In addition divalent magnesium is a more effective inhibitor at lower concentration than monovalent sodium. Similarly, it has been shown that Mg^{++} inhibits intercalation of the *cis*-porphyrin (Figure 8) over the same concentration range. These data support the contention that the photosensitizer must be in close proximity to DNA for maximum photosensitization activity.

Figure 8. Agarose gel electrophoresis of circular DNA relaxed by topoisomerase I in the presence of porphyrin and $MgCl_2$. Lanes 1–8 contained 2 μ M *cis*-dicationic porphyrin and: 25, 5, 1, 0.5, 0.2, 0.1, 0.05, 0.01 mM $MgCl_2$ respectively. The porphyrin and the $MgCl_2$ were added after the first and before the second stage of the assay. The entire assay and electrophoresis were carried out in the absence of light.

DISCUSSION

Measuring the relaxation of closed circular DNA by topoisomerase I is a simple and rapid way to determine whether a DNA-ligand binds by intercalation. It is especially useful when dealing with ligands having limited water solubility and/or intense fluorescence as in the case of the diphenyl porphyrins studied in this work.

The results obtained here confirm those of Sari, et al.(7) in demonstrating that T4MPyP, tricationic porphyrin and the *cis* and *trans* dicationic porphyrin all bind to DNA by intercalation. The monocationic porphyrin shows no evidence of intercalation under the conditions used here. It has also been shown that intercalation decreases with increasing ionic strength. Note, however, that the assay does not measure non-intercalative binding and the extent to which intercalation versus outside binding may contribute to photosensitized strand breaks in DNA has not been established.

Cis and *trans* dicationic porphyrin appear to be very active photosensitizers producing extensive strand scission in DNA at relatively low doses of porphyrin and light. Under the same conditions used here, T4MPyP, tricationic porphyrin, monocationic porphyrin, hematoporphyrin derivative and methylene blue produce significantly less strand breaks (data not shown).(18,22) The reason for the high level of activity noted for the *cis* and *trans* dicationic porphyrin is not known; however, it cannot be simply related to the strength of the binding since the apparent binding constant for the *cis* and *trans* diphenyl is approximately 8-fold smaller than that measured for TYMPyP.(7) Other explanations include the photophysical properties of the porphyrins, base specificity of binding and the local conformation of the ligand-DNA complex. All of these factors may play a role in the efficiency of the photosensitized induction of strand breaks.

The nuclease-like effect of visible light in the presence of the dicationic porphyrin results in progressive nicking of the circular plasmid. Extensive nicking is needed before linear fragments appear indicating that the lack of bias toward producing new nicks on opposite strands near complimentary nicks. It could be that uninterrupted double strands are preferred for porphyrin binding which, in the presence of light, causes a new single-strand DNA break sufficiently distal to the previous breaks to prevent linearization of the circular DNA. In addition, the extensive

single-strand breaks in the circular DNA increases its electrophoretic mobility. This may be due to multiple nicks causing increase flexibility. The change in electrophoretic mobility as a result of photodamage may be a function of size of the DNA with small molecules more effected than larger molecules. DNA damaged by treatment with various ligands has been shown to be a poor substrate for exonuclease activity.(20,21) Either the polynucleotide end contains an adduct or has been altered so as to interfere with exonuclease cleavage. In this study, DNA damage mediated by light in the presence of these porphyrins confers resistance to restriction endonucleases. This resistance is only partial but increases with the degree of damage. We suspect that only a fraction of the molecules, which increases with increased dose, are damaged at the restriction endonuclease specific hexanucleotide site. It may be that single-strand breaks within the restriction endonuclease site interferes with enzyme activity towards the complementary strand. In contrast, damaged DNA is an excellent substrate for the nicking specific S1 endonuclease presumably due to the photo-induction of multiple nicks. This suggests that the nature of the nick on the complementary strand is not critical to S1 cleavage of the complementary strand in that an end other than a 3' OH and a 5' P is generated by the photodamage. It will be informative to determine the precise nucleotide sequence at which the cleavage occurs and compare it to other cationic porphyrins.

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