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DNA photocleavage by novel intercalating 6-(2-pyridinium)phenanthridinium viologens

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Abstract A new type of DNA-intercalating *viologen* dications, derived from the *N,N'*-dialkyl-6-(2-pyridyl)phenanthridine structure (in which dialkyl is $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, or $(-CH_3)_2$, abbreviated dq2pyp, dq3pyp, and Me2pyp, respectively), are able to produce frank strand breaks in supercoiled plasmid DNA upon irradiation with visible light. The amount of photocleavage is similar for the three drugs. The observed DNA photosensitization appears to follow a single-strand cleavage model, as shown by a kinetic analysis of the reaction with dq2pyp. The photodynamic action of the drugs seems to be initiated by a light-induced electron transfer reaction from the nucleobases, given the singlet excited-state redox potentials (ca. + 2.1 V vs. SHE) and the low quantum yields of singlet molecular oxygen production of the drugs (0.1–0.2 in aerated D_2O).

Key words: Intercalating viologen; DNA photocleavage; Photoinduced electron transfer

1. Introduction

There is a growing interest in the rational design of novel small molecules able to react with nucleic acids and/or to probe their rich polymorphism, in order to improve the armory of molecular tools for detecting and manipulating the genetic material [1]. Photocleavage of nucleic acids is a valuable property of DNA-targeted drugs, since it allows to use light as a trigger of the nuclease activity. By attaching the drug to an appropriate oligonucleotide, sequence-selective photonucleases can be created, directed to both single-stranded or double-stranded nucleic acids [2]. Photosensitization of DNA by drugs may be clinically useful as well; i.e. photoaddition of psoralens to DNA is used to treat psoriasis and other skin diseases.

Chemically different compounds have been reported to photocleave DNA/transition-metal coordination compounds (e.g. 1,10-phenanthroline derivatives of Ru(II), Rh(III) or Co(III)), polycyclic heteroaromatic dyes (such as riboflavin, rose bengal or ethidium), and ketones (acetophenone, benzophenone) [3]. Three main mechanisms have been proposed to explain such an activity [3]: (a) singlet oxygen ($^1O_2$) generation by energy transfer from a drug triplet to O_2, followed by subsequent dark reaction of $^1O_2$ with guanine; (b) direct or $OH^•$-mediated hydrogen abstraction from the deoxyribose moiety; (c) oxidation of guanine by the photoexcited drug. It is not uncommon to find the simultaneous participation of two of such processes in the sensitized photooxidation of nucleosides [4].

In a recent paper [5], we have reported the intercalative interaction with DNA of a novel family of photoactive organic compounds, namely dialkylated 6-(2-pyridyl)phenanthridine (pyp) *viologens* (Scheme 1). The possibility of photochemistry with DNA was then suggested, based on their singlet excited state redox potentials and the efficient fluorescence quenching observed with GMP and AMP ($k_q = 1.2 \times 10^{10}$ and 6.8×10^9 $M^{-1} \cdot s^{-1}$, respectively). The goal of the present paper is to report the capability of dq2pyp, dq3pyp, and Me2pyp, to photosensitize DNA cleavage, as well as to obtain evidences about the possible mechanism responsible for their photodynamic activity.

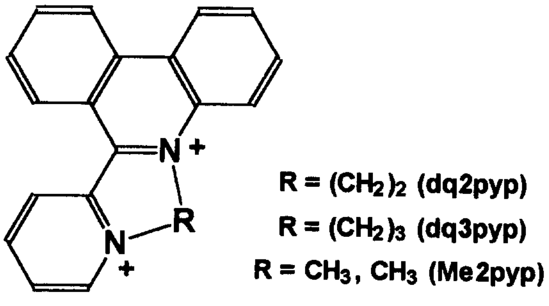
2. Materials and methods

2.1. Materials

Supercoiled pBR322 plasmid DNA was purchased from New England Biolabs. Calf thymus DNA was isolated in our laboratory following the procedure of Kay et al. [6], and further treated with RNAse, pronase, and proteinase K in order to thoroughly eliminate contaminating RNA and proteins. The three drugs were prepared in our laboratory, and their synthesis will be described elsewhere (Orellana et al., to be published). Molecular weight marker X, *Eco*RI, catalase and superoxide dismutase, were from Boehringer-Mannheim. Sodium azide was from Aldrich. All the experiments were conducted in 'P' buffer (10 mM sodium phosphate, pH 5.5).

2.2. Photosensitized cleavage of pBR322 plasmid DNA

Samples for irradiation (15 μl, in Eppendorf tubes) contained P buffer and variable amounts of plasmid and drugs. Irradiation was made via a 6.5-mm diameter fused-silica fiber optics (Fiberguide Ind.) fitted to the tube, that delivered >360 nm light from an Oriel 150-W Xe lamp provided with IR and cut-off filters. After exposure to light, 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose) were added to the reaction mixtures. All the samples were



Scheme 1.

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Abbreviations: dq2pyp, 6,7-dihydro-pyrido[2′,1′:3,4]pyrazino[1,2-*f*]phenanthridinediium dibromide dihydrate; dq3pyp, 7,8-dihydro-6*H*-pyrido[2′,1′:3,4][1,4]diazepino[1,2-*f*]phenanthridinediium dibromide hydrate; Me2pyp, 6-(1-methylpyridine)-5-methylphenanthridinediium dichloride hemihydrate; $^1O_2$, singlet molecular oxygen.

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analyzed by 1% agarose gel electrophoresis in Tris-acetate (TAE) buffer, followed by 30 min of ethidium bromide staining (1 mg/ml). Once electrophoresed, all the stained gels were photographed on an UV transilluminator using a Polaroid camera with an orange filter. The film negatives (Polaroid 665) were scanned with a Molecular Dynamics Model 325S densitometer and the spots were integrated using the Image- Quant software. In order to compare different lanes of the same or different gels, a normalization procedure was adopted: the intensity of each spot was divided by the sum of the intensities of all spots in the same lane. The lesser intercalation of ethidium into supercoiled DNA (form I) was taken into account by dividing the corresponding intensity by 0.8 [7]. The plasmid used throughout showed a dimeric form (D) in the agarose gels, which appeared as the slowest spot in each lane. As cleavage proceeded, the amount of dimer decreased and eventually disappeared before any linearized form (III) was formed. This fact indicates a direct transformation D→II (nicked form), which must be taken into account when quantifying the cleavage. Thus, the photocleavage extent (in the absence of form III) was defined as [II]/([I] + [II] + [D]).

2.4. Photobleaching studies

Samples (1 ml, in quartz cuvettes) containing P buffer, dq2pyp (2×10^{-5} M) and, when required, calf thymus DNA (5×10^{-4} M base pairs), were irradiated for different times using the same conditions as in the photocleavage experiments but removing the optical fiber. Absorption spectra were recorded between irradiations, in order to detect light-induced chemical changes in the drugs.

2.5. Determination of the quantum yield of singlet oxygen production ($\Phi_{^1O_2}$)

The steady-state $^1O_2$ luminescence at 1270 nm was used to quantify the yield of $^1O_2$ formation in air-equilibrated samples of dq2pyp and dq3pyp in D_2O (Euriso-top, > 99.8% D), using the spectroscopic system described before [8]. The estimated error of the measurements is 3%. Rose bengal in D_2O ($\Phi_{^1O_2} = 0.75$ [9]) was used as reference sensitizer.

3. Results and discussion

Fig. 1A depicts the photocleavage of supercoiled pBR322 plasmid by the three investigated viologens, dq2pyp, dq3pyp and Me2pyp. When the plasmid is irradiated in the presence of the drugs, the intensity of the nicked form band (II) increases, whereas that of both form I and the dimeric form (D) substantially decreases. Under the conditions used, only single strand breaks are produced (see the control lane 2 of plasmid linearized by *Eco*RI). Given the experimental error, a quantification of the photoreaction extent with the three drugs under the conditions of reported in Fig. 1A yields similar amounts of cleavage: 42%, 42%, and 59% for dq2pyp, dq3pyp and Me2pyp, respectively. It should be stressed that the irradiation time of each drug in the presence of plasmid was inversely proportional to the area under their corresponding absorption spectra at wavelengths > 360 nm [5]. When the irradiation times and drug concentrations are the same, the photodynamic activity is in the order: dq2pyp > dq3pyp > Me2pyp; thus, all the kinetic and mechanistic experiments described below were conducted with dq2pyp. A similar amount of cleavage is to be expected if the photosensitization results from a base oxidation process, since the redox potential of the singlet excite state of the three drugs is similar, and (as in the conditions of the experiment) if the three DNA saturations are also similar.

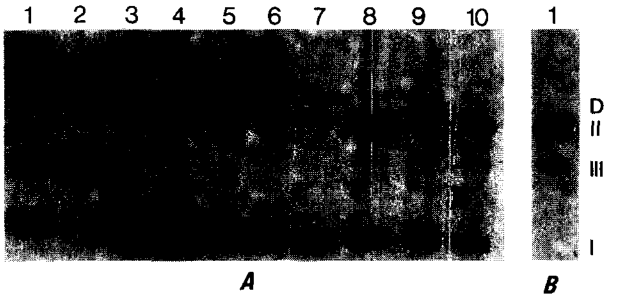

Fig. 1. (A) Photocleavage of DNA by 6-(2-pyridinium)phenanthridinium viologens. Plasmid DNA (pBR322, 6.94×10^{-5} M in bp) irradiated in the absence/presence of dq2pyp, dq3pyp, and Me2pyp (10 μM). Lane 1 = molecular weight marker X (Boehringer-Mannheim); lane 2 = pBR322 linearized with *Eco*RI; lane 3 = pBR322 in the dark; lane 4 = pBR322 irradiated for 90 min; lanes 5–7 = pBR322 + dq2pyp, dq3pyp, and Me2pyp, respectively, in the dark; lanes 8–10 = pBR322 + dq2pyp, dq3pyp, and Me2pyp, irradiated for 30, 40 and 90 min, respectively. Key: I = supercoiled DNA; II = relaxed plasmid; III = linearized plasmid; D = plasmid dimer. (B) Linearization of pBR322 by photocleavage with dq2pyp. In these conditions (pBR322 4×10^{-5} M, dq2pyp 33 μM, 30 min of irradiation) both I and D forms have disappeared, and forms II and III have been produced.

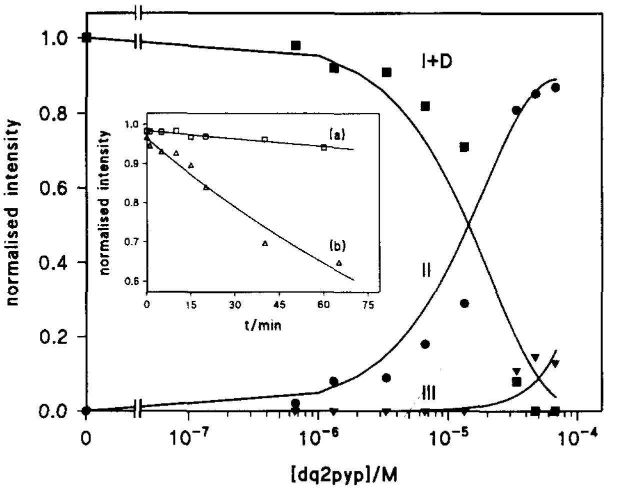

Fig. 2. Plot of the intensities for forms I + D, II and III vs. dq2pyp concentration upon irradiation for 30 min in P buffer. The fits correspond to the single-strand cleavage model of Kishikawa et al. [10], resulting in $k_{I\rightarrow II} = 1600\ M^{-1} \cdot min^{-1}$ and $k_{II\rightarrow III} = 65\ M^{-1} \cdot min^{-1}$. *Inset*: evolution of form I intensities with time before any form III has been produced, for dq2pyp (a) 3.3 μM, and (b) 13.3 μM, fitted to a single-exponential function. The $k_{I\rightarrow II}$ values obtained from the fit are (a) 1100 $M^{-1} \cdot min^{-1}$ and (b) 2000 $M^{-1} \cdot min^{-1}$, very similar to that estimated from the plot of intensities vs. drug concentration.

Illumination of dq2pyp intercalated into an excess of calf thymus DNA shows no spectral changes in the visible region of the drug up to 4 h. On the contrary, in the absence of DNA there is a small amount of drug photobleaching, probably due to light-activated nucleophilic attack by the solvent or the phosphate groups. It appears that DNA protects the intercalated drug against photodegradation; indeed, molecular modeling [5] showed that the pyridinium moiety (the most susceptible to such an attack) contacts the wall of the DNA minor groove, avoiding the access of nucleophilic agents. Additionally, the spectral constancy also indicates that no photoadducts form between dq2pyp and DNA.

Since form III was evident only at relatively long irradiation times and/or high sensitizer concentrations (Fig. 1B), the absence of an independent pathway for double-strand cleavage is proposed: linearization would be produced by accumulation of single strand breaks at close positions in the opposite strands. In order to confirm this point, a coupled kinetic analysis of the cleavage by dq2pyp was conducted, using the model and equations put forward by Kishikawa et al. [10]. Fig. 2 reflects the amount of cleavage as a function of both drug concentration and time, together with the best fits of the single-strand cleavage model [10] to the experimental data. From the analysis, a $k_{\mathrm{I}\rightarrow\mathrm{II}}/k_{\mathrm{II}\rightarrow\mathrm{III}}$ ratio of kinetic constants of about 25 results, indicating that linearization occurs more probably by accumulation of single-strand breaks. Moreover, attempts to fit a double-strand cleavage model [10] to the experimental data resulted in unacceptable results. Thus, dq2pyp seems to photocleave DNA essentially by producing randomly distributed single-strand breaks.

The involvement of diffusible oxygen species in the DNA photosensitization mechanism was also investigated by irradiating the dq2pyp/plasmid samples in the presence of 10^{-3} M sodium azide (an efficient singlet oxygen quencher), superoxide dismutase (SOD, catalyst of the $2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$ reaction), catalase (catalyst of the $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$ reaction), and a mixture of SOD and catalase. Except with catalase alone, where no changes are detected, in all the cases a *slight* decrease of the cleavage was observed. SOD (with and without catalase) showed the largest inhibition, although it was constant even at enzyme concentrations up to 3.5 mg/ml, suggesting a secondary role of $O_2^{-}\cdot$ in the photocleavage mechanism. The smaller effect of sodium azide also points out a minor involvement of singlet oxygen; this interpretation is further supported by the measured low quantum yields of $^1O_2$ formation in D_2O: 0.22 and 0.10 for dq2pyp and dq3pyp, respectively. Moreover, an even lower production of $^1O_2$ is expected when the drug is intercalated into DNA, since molecular oxygen would have a lesser access to it [11].

Taking into account the redox potential of the singlet excited state of the three drugs (+ 2.1 V vs. SHE [5]) and those of the DNA bases (−1.05, −1.15, −1.25, and −1.40 vs. SHE, for guanine, adenine, thymine and cytosine, respectively [12]), a photoinduced electron transfer from the DNA bases (preferably guanine) to the excited drug is thermodynamically feasible. The occurrence of such a charge transfer has been demonstrated indeed recently using laser flash photolysis and spectroelectrochemical measurements [13], and is responsible for the previously observed quenching of the drug fluorescence by mononucleotides and DNA. The oxidized base would then evolve towards a break in the sugar-phosphate backbone in one of the ways previously described [4]. This is not without precedent: it has been found that the singlet excited state of methylene blue ($E^{*+/0} = +1.8$ vs. SHE) is efficiently quenched in GC rich (but not in AT rich) regions of DNA to yield photosensitization, although a $^1O_2$ mechanism cannot be ruled out [11]. This might be also the case of the photocleaving viologen MDAP^{2+} (*N,N'*-dimethyl-2,7-diazapyrenium dication), which has been proposed to photooxydize DNA [14]; other authors claim, however, that its photonuclease activity is due exclusively to $^1O_2$ generation [15].

Future experiments will aim to study the photocleavage of different nucleic acid structures (triple helices, single-strands, hairpins, etc.), in order to investigate the structural specificity of these novel artificial photonucleases.

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