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Photoinduced cleavage of plasmid DNA in the presence of pterin

Carolina Lorente[a], Andrés Héctor Thomas[a], Laura Sofía Villata[a], Daniela Hozbor[b], Antonio Lagares[b] and Alberto Luis Capparelli[a]

(a) Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Casilla de Correo 16, Sucursal 4, (1900), La Plata, Argentina. E-mail: alcappa@dalton.quimica.unlp.edu.ar ; (b) Instituto de Bioquímica y Biología Molecular (IBBM), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata. 47 y 115 (1900) La Plata, Argentina. E-mail: lagares@nahuel.biol.unlp.edu.ar

Summary

The photoinduced cleavage of plasmid DNA by UV-A light in the presence of pterin was investigated. Electrophoretic analysis of the irradiated plasmid pUC18 in the presence of pterin showed that UV light of 350 nm induced the transformation of a significant proportion of the supercoiled plasmid to its relaxed form. A minor proportion of plasmid forms are also converted to the linear plasmid isomer at the longer irradiation times. All these transformations during irradiation can be observed in the absorption spectrum of DNA as function of time. Such spectral modifications correlated with the extent and the kinetics of plasmid relaxation, but not with the appearance of the linear plasmid. None of these changes were operative without the irradiation with UV-A light. Control experiments with pterin or plasmid DNA irradiated separately, showed no photochemical changes. Results taken together suggest that the observed changes in the supercoiled plasmid as well as the spectral modifications both derive from the generation of single-strand break in the DNA.

Introduction

As reported in the literature, pterins have been suspected as photoinducer of genetic alterations. The importance of this effect is mainly associated with the broad band absorption at wavelength larger than 320 nm. This fact makes relevant the effect of solar irradiation in the UV-A region on those biological systems where pterins could be accumulated. This suggestion was advanced in the last eighties (1).

Several studies are reported in the literature describing the interaction of DNA with UV radiation. Dimers of pyrimidine are normal photoproducts resulting from this interaction (2). Such changes were observed in vitro and also in living organisms when DNA is exposed to UV-B (290-320 nm) radiation. Chemical changes in DNA induced by UV light result in mutagenic and carcinogenic alterations. These changes induce molecular processes competing with those involved in enzymatic reaction for repairing the DNA damage. If the later process is low enough, a genetic failure will be transmitted to the new cell (3). It has been shown that UV-B radiation is able to induce several types of skin cancer (4).

Experiments with cells showed that UV-A light (320-380 nm) is also mutagenic and carcinogenic (5,6). However, DNA does not present detectable absorption in this wavelength range. These results are of biological significance because UV-A is mainly present in the solar radiation reaching the earth surface. Indirect processes may produce genetic damage induced by UV-A radiation. In fact, photoactivation of a second molecule may start a series of processes when interacting with DNA, even its denaturation and/or fragmentation (7).

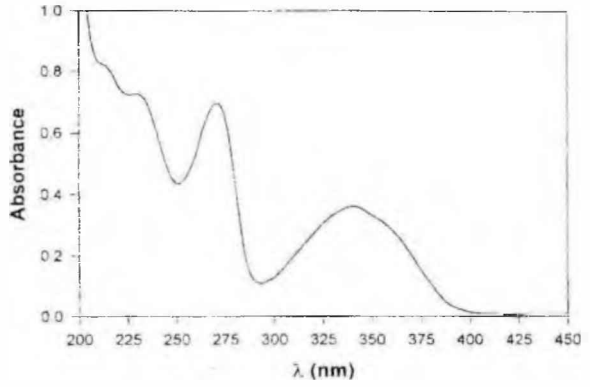
Previous reports support these photoactivated processes involving the participation of other molecules that are photoactive in this wavelength region leading to the DNA and RNA photocleavage. These molecules can be referred as "photocleavers" or "photocleavage agents" (7), which can be organic compounds, such as anthraquinones (8,9), dibenzoyldiazomethane (10), 2-aminoimidazolone (11), 6-(2-pyridinium)phenanthridinium (12), porphyrins (13) or metal complexes (7), particularly those of ruthenium, vanadium, copper, cobalt. Several of these complexes have been proposed as model probes in photocleavage studies.

Correspondence to: Alberto Luis Capparelli,
Casilla de Correo 16, Sucursal 4, (1900), La Plata, Argentina.

As shown in figure 1, pterin absorbs UV-A light. Some biomedical studies suggest that pterins are involved in [illegible] the skin's pigmentation, which depend on the [illegible] these compounds in the cell (14). Moreover, a recent study showed that pterin, 6-carboxypterin and folic acid are able to induce the hydroxylation of deoxyguanosine in calf thymus DNA (15). In the light of these facts, it is interesting to look after the potential effects of these compounds as putative photoinducer of skin cancers in living organisms.

In this paper we report *in vitro* experiments performed on supercoiled PUC18 plasmid DNA and analyze the extension of alteration and even damage photoinduced by pterin.

Fig.1: Absorption spectrum of aqueous solution of pterin 6.8×10^{-5} M at pH 6.5 and 25°C.



Materials and methods

Materials

pUC18 plasmid DNA was isolated in our laboratory from Escherichia coli DH5a (pUC18) using the alkaline lysis procedure according to (16) and further treated with RNAase (from Sigma) and phenol (from Carlo Erba)-tris in order to eliminate contaminating RNA and proteins. A final step of isopropanol precipitation was carried out to concentrate DNA in sterile aqueous solutions. The absorbance ratio A^{260}/A^{280} was used as a purity control of the sample. In all our samples, this ratio was ≈ 2, being that indicative of a low or almost negligible protein content in the starting DNA.

EcoRI (from Promega) was used for linearizing the plasmid forms. The experimental conditions employed are reported elsewhere

Pterin p.a. (Sigma), L-histidine (from Fluka) and methanol (from Merck) were used without further purification.

Experiments were performed in solutions free of oxygen (in atmosphere of N_2 free of O_2 or He), in presence of air and saturated with O_2 ultrapure, respectively.

Photochemical studies

Experiments were performed under continuous irradiation in quartz cells of 0.6 ml and 0.2 cm optical path. Rayonet lamps RPR (3500 Å) provided by Southern N.E. Ultraviolet Co. were employed. The pH of DNA and pterin solutions was adjusted to 6.5 before irradiation. The whole experiments were carried out at 25 °C. Further experimental details are given in figure legends.

Absorption spectra of solutions were taken after a few seconds till 2 hours of photolysis. Spectral changes of irradiated DNA and pterin were analyzed in the range 200-450 nm. UV-Visible spectra were performed on a Cary 3 (Varian) spectrophotometer, using a program for smoothing and averaging signals.

Control experiments showed that the spectrum of DNA plus pterin before irradiation resulted exactly the additive combination of the individual spectra of pterin and DNA (not shown). No changes in the absorption spectrum of DNA were observed, even after 120 minutes of continuous irradiation. Moreover, no significant changes in the absorption spectra of pterin solutions were observed during 120 minutes of irradiation.

Electrophoretic runs

Electrophoretic runs of control and irradiated plasmid samples were performed in 0.8 % agarose gels using TBE buffer (0.045 M Tris-borato/0.001EDTA). DNA was visualized with ethidium bromide (Sigma, 1mg/ml) under UV illumination.

Three major forms of plasmid DNA can be detected. The form with a higher mobility corresponds to a supercoiled structure whereas the second one should be a more relaxed form. The third band corresponds to dimeric species of plasmid DNA. Intensities on the gel are proportional to the amount of DNA. When DNA containing solutions are treated with the restriction enzyme *Eco*RI, all the forms present are transformed to a plasmid form presenting only one electrophoretic band. This band corresponds to a linear form as expected.

FOTODYNE recorder with a UV transiluminator and a POLAROID camera were employed. The film negatives were scanned in a Hewlett Packard scanner. The intensity spots were integrated using the Sigmagel (Jandel Co) program. In order to compare different lanes of the same gel, a normalization procedure was adopted: the intensity of each spot was divided by the sum of the intensities of all spot in each lane. The lesser intercalation of ethidium into supercoiled DNA was taken into account by dividing the corresponding intensity by 0.8 (17).

Results

Electrophoretic analysis of plasmid pUC18 irradiated with UV-A light in the presence of pterin.

To investigate the characteristics of the photoinduced cleavage of DNA by pterin, samples of plasmid pUC18 were analyzed by agarose gel electrophoresis after different irradiation times using UV-A light at 350 nm. As shown in Figure 2, the DNA solutions irradiated in the presence of pterin showed an increase in the relaxed plasmid form concentration and a simultaneous reduction in the supercoiled form concentration. In addition to these observations, a new plasmid form was detected at longer than 60 minutes irradiation times. This new form presents an electrophoretic mobility coincident with that of pUC18 linearized form obtained after digestion with restriction enzyme EcoRI. No electrophoretic changes were observed in plasmid DNA solutions containing pterin and kept in the dark several hours. Control experiments using plasmid solutions without pterin did not show any electrophoretic change after irradiation.

Fig.2: Electrophoretic runs. ***Lane 1:*** solution of pterin ($1x10^{-5}$ M) and plasmidic DNA ($3.8x10^{-5}$ M in bp) irradiated during 140 minutes. ***Lane 2:*** control of non irradiated DNA solution of equal concentration. ***Lane 3:*** solution of lane 1 before irradiation. ***Lane 4:*** pattern of the enzymatically linearized plasmid.

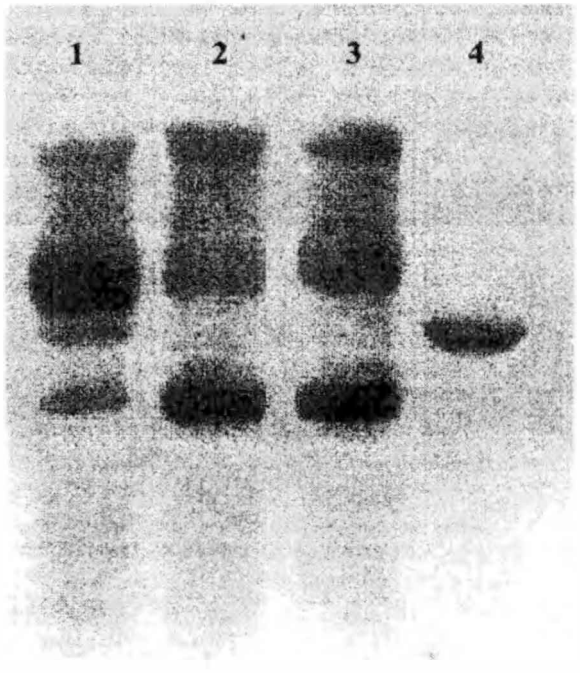
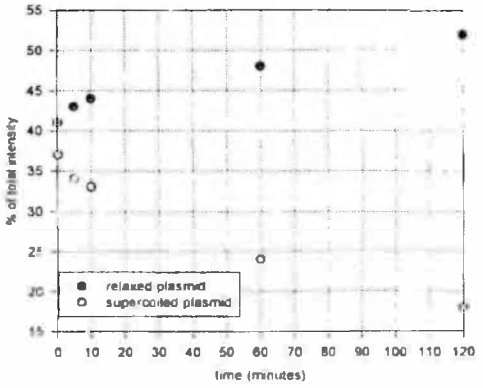


Figure 3 shows the time evolution of relative concentrations of relaxed and supercoiled plasmid forms in irradiated solution of plasmid pUC18 in the presence of pterin. The increase in the relaxed form concentration correlated well with the decrease in the supercoiled form concentration.

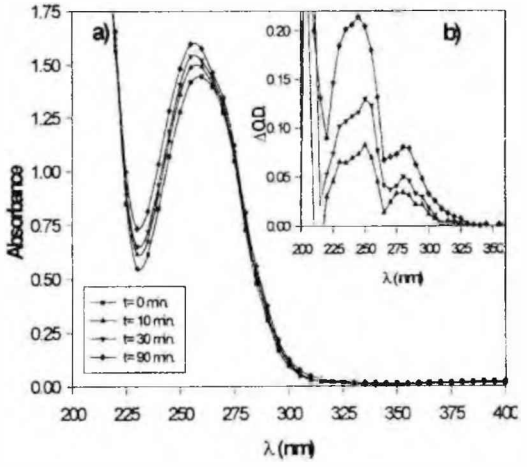
Fig.3.: Time evolution of relative concentrations of plasmid forms in irradiated solution of plasmid pUC18 ($3.8x10^{-5}$ M in bp) in the presence of pterin ($1x10^{-5}$ M) Intensities of supercoiled plasmids were corrected as reported in the literature (17).



Spectral changes:

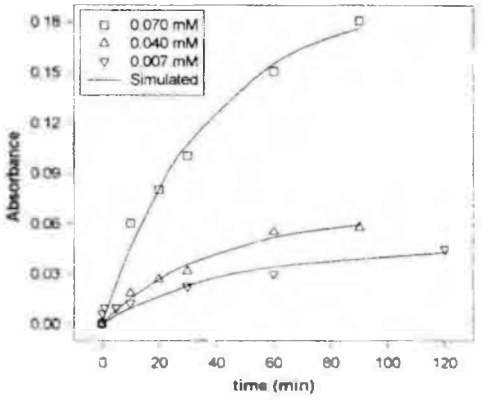
To investigate the spectral characters of plasmid pUC18 before and after irradiation with UV-A light in the presence of pterin, the spectra were recorded at different irradiation-times (0-120 minutes) and plotted as shown in Figure 4. DNA spectra changed in time, with an increase in the absorbance in the range of 220 to 300 nm.

Fig.4: (a) Evolution of the absorption spectra of solutions of plasmid DNA ($3.8x10^{-5}$ M in bp) irradiated in the presence of pterin ($1x10^{-5}$ M), as a function of irradiation time. In this Figure, the spectrum of a ($1x10^{-5}$ M) pterin solution was subtracted to the experimental spectra recorded at the different times. (b) Difference spectra obtained after subtracting to the spectra at different times the corresponding one at t=0.



In another set of experiments, shown in Figure 5, the variation of absorbance at 260 nm along the first two hours of irradiation using different pterin concentrations was analyzed.

Fig.5: Absorbance changes at 260 nm of plasmid DNA solution (5x10-5 M in bp) after irradiation at different pterin concentrations. Solid lines are obtained from fitting a first order law (eq. 1) using the parameters listed in Table 1.



A first order rate law was found to describe the changes in absorbance (A_t-A_o) according to the following equation:

$$(A_t - A_o) = (A_\infty - A_o).[1-\exp(-k.t)]. \qquad (1)$$

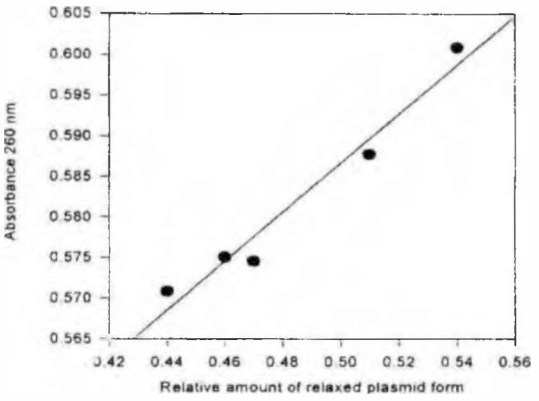
Kinetic constants are presented in table 1. The first order rate constant was found independent on the pterin concentration as expected. Solid lines in Figure 5 were calculated with this set of parameters.

Tab.1: Apparent first order rate constants at different pterin concentrations.

[Pt]/mM	$(A_\infty - A_o)$	k/min
0.007	0.04	0.0225
0.040	0.06	0.0259
0.070	0.18	0.0269

Interestingly, the extent of the spectral changes presented a linear correlation with the increase of the relaxed plasmid form (Figure 6).

Fig.6: Correlation between the absorbance changes and the relative amount of the relaxed form. Both, absorbance changes and electrophoretic runs match in time. The relative amount of relaxed form is expressed as I_r/I_o, where I_o means the "total amount" of DNA estimated by adding the intensities of the different spots in each lane and I_r corresponds to the intensity measured for the relaxed form in the same lane.

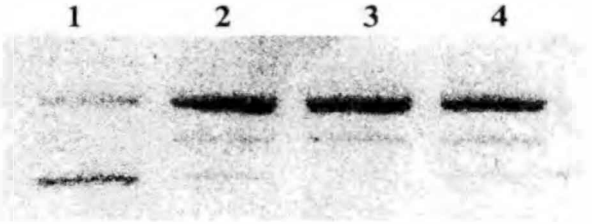


No spectral changes were observed in a solution, made by mixing pterin and DNA, kept in the dark during a few hours. When pterin or DNA solutions were separately irradiated, their absorption spectra do not alter. In the same way, the absorption band of pterin in the range 300-450 nm (Figure 1) does not present alterations after the irradiation even in the presence of DNA at this pH.

Analysis of plasmid photolysis in the absence of oxygen and in the presence of selective scavengers.

Experiments carried out in the absence of dissolved oxygen showed comparable results to those performed in either air equilibrated or oxygen saturated. All experimental conditions led to a similar increase in the amount of relaxed DNA plasmid. (Figure 7). The result strongly suggests that molecular oxygen would not be involved in the photolytic cleavage of DNA by pterin, here characterized.

Fig.7: Effect of the oxygen concentration on the photoinduced cleavage of plasmidic DNA ($3.8x10^{-5}$ M in bp) in the presence of pterin ($1x10^{-5}$ M) ***Lane 1:*** non irradiated solution. ***Lanes 2:*** irradiated solution in absence of O_2. ***Lanes 3:*** saturated in O_2 irradiated solution. ***Lanes 4*** aerated irradiated solution. The irradiation time was 120 minutes.



In addition plasmid cleavage was not inhibited in the presence of 1.2 mM histidine, a singlet oxygen scavenger. Similarly, DNA cleavage was observed in 1.7 mM methanol, an active quencher agent of hydroxyl radical (18).

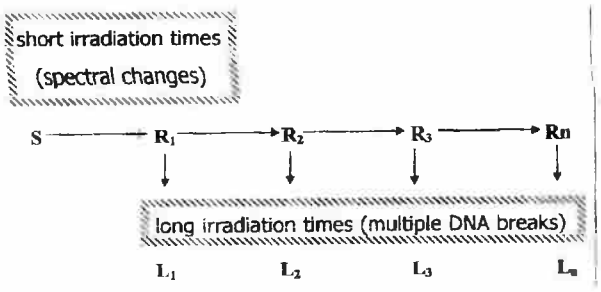
Discussion

Here we analyzed the photolysis of a plasmid substrate to characterize the kinetics of the proccess. According to evidence from our laboratory pterin-6-carboxylic acid and folic acid but not pterin are photolyzed under UV-A illumination. While radical intermediates are detected in the first stage of the photolysis of folic acid (19), pterin-6-carboxilic acid photolyses with the release of CO_2 (20). Thus, it is not straight forward the assignment of an active role in DNA damage to pterin-6-carboxilic acid derivatives themselves. To circumvent this problem we used pterin as photocleaver agent.

Results show that irradiation of DNA in the presence of pterin leads in the short time (minutes) to a continuous increase of the DNA absorbance at 260 nm (A^{260}). Changes in the A^{260} follow first order kinetics and a parallel increase in the circular relaxed plasmid form takes place. These observations strongly support the possibility that spectral changes resulted from the conversion of the supercoiled plasmid to its relaxed form after a single strand break within the DNA helix. This interpretation is in agreement with the observed linear dependence between the A^{260} and the amount of the relaxed plasmid form, each of them sampled at identical irradiation times (Figure 6). The appearance of the linear plasmid form at long irradiation times might be due to a structural requirement of at least two close breaks in the opposite DNA strands.

Results suggest that early changes in the spectral absorption of the DNA after irradiation as well as those changes observed later in the electrophoretic pattern are consequence of cumulative breaks in both DNA strands (Figure 8).

Fig.8: Time sequence of spectral and electrophoretic changes during pUC18 irradiation. S: supercoiled plasmid; Ri: relaxed plasmid; Li: linear plasmid; i: breaks number.



Assuming a random model for the location of the UV-A photoinduced breaks within the plasmid sequence, the probability that a given break (n) could result in a linear form should increase with n. Although accumulation of many breaks along the linear plasmids could result in plasmid fragmentation at long irradiation times. We have no experimental evidence of such phenomenon.

Regarding the type of mechanism by which pterin participates in the photocleavage of DNA, no evidence was yet obtained to support a direct interaction pterin-nucleic acid. Photoexcited pterins lost energy through radiative and non-radiative pathways. The observation that pterin absorption does not change in intensity or shape after the mixture with DNA, particularly in the region of $\lambda > 320$ nm, suggests that pterins do not directly bind to DNA or that their binding affinity is too low to be detected under our experimental conditions.

There are several mechanisms of interaction between DNA and photocleavers. At least, three different groups of processes have been recognized: (1) the photocleavager can abstract hydrogen atoms from the deoxyribose groups of DNA without the requirement of binding between the nucleic acid and the photocleaver, (2) generate a diffusible intermediate (singlet oxygen or hydroxy radicals) that reacts with the nucleic acid to initiate a nonselective strand cleavage of the DNA or (3) operate by single electron-transfer through a process that requires the intercalation of the photocleaver. In the reaction described in this work, the observed photocleavage in the absence of disolved oxygen rules out the participation of singlet O_2 to induce the DNA damage (Figure 2b). This observation is further supported by similar results obtained in the in presence of L-hystidine, an agent that captures singlet O_2 species. Using independent evidence it has been previously suggested that singlet O_2 plays little role, if any, in calf thymus photoinduced DNA damage by pterins (15). The DNA damage obtained in the presence of methanol during the irradiation also suggests that OH radicals would not participate in the photolysis.

Acknowledgements

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