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Loss of transforming activity of plasmid DNA (pBR322) in *E. coli* caused by singlet molecular oxygen

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Plasmid DNA pBR322 in aqueous solution was exposed to singlet molecular oxygen ($^1O_2$) generated by microwave discharge. DNA damage was detected as loss of transforming activity of pBR322 in *E.coli* (CMK) dependent on the time of exposure. DNA damage was effectively decreased by singlet-oxygen quenchers such as sodium azide and methionine. Replacement of water in the incubation buffer by D_2O led to an increase in DNA damage. 9,10-Bis(2-ethylene)anthracene disulfate was used as a chemical trap for $^1O_2$ quantitation by HPLC analysis of the endoperoxide formed.

Singlet oxygen; DNA damage; Plasmid pBR322; DNA

1. INTRODUCTION

Radiation-induced and much of the chemically induced DNA damage is attributed to free-radical reactions, involving notably the hydroxyl radical [1,2]. However, non-radical reactions of electronically excited species are also important, e.g. in DNA damage by photooxidation, and can explain the effects of photosensitizers which generate singlet molecular oxygen $(^1\Delta_g)O_2$. $^1O_2$ was demonstrated recently by its monomol emission in tetracycline photosensitization [3] and enzymatically with the chloroperoxidase reaction [4], and by its dimol emission in enzyme reactions such as cyclooxygenase [5]. Although $^1O_2$ has long been known to react with constituents of nucleic acids [6,7], there has been uncertainty as to its importance in eliciting DNA damage. Using a physical source of $^1O_2$ by employing a microwave discharge system as described by Ogryzlo [8], we examined the transforming activity of the plasmid pBR322 in *E. coli*. Taking care to exclude O atoms and O_3, it is shown that $^1O_2$ leads to a loss of biologically active DNA. The increase of $^1O_2$'s effectiveness in deuterium oxide and the effects of some singlet-oxygen quenchers underscore the importance of $^1O_2$ in biological processes of DNA damage.

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2. MATERIALS AND METHODS

2.1. *Microwave discharge system for generation of singlet molecular oxygen*

The generation of singlet molecular oxygen $(^1\Delta_g)$ in the gas phase was performed according to Ogryzlo [8] using an electrodeless microwave discharge in a low-pressure (14 Torr, flow 0.7 l/min) stream of oxygen. The tuned cavity, cooled with compressed air, was connected with a 2450 MHz microwave generator (Microtron 2000, microwave power generator MK2, EMS), run at 150 W.

Elimination of O atoms in the microwave discharge system: As described by Ogryzlo [8], the persistence of O atoms in the gas stream can be prevented by maintaining a certain mercury gas

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pressure in the system and, secondly, by a mercuric oxide layer on the glass tubing. Thus, a mercury reservoir was incorporated between the inlet needle valve and the quartz tube. During operation a ring of mercuric oxide developed about 3 cm beyond the discharge region. The temperature of the mercury was maintained at 45°C. A cooling trap (dry ice/acetone) then prevented mercury from reaching the reaction vessel. Upon addition of small amounts of NO through an inlet valve behind the microwave discharge region, as a sensitive indicator for O atoms by emission of white light through NO_2^* chemiluminescence, no light emission could be observed with the dark-adapted eye under conditions identical to those employed for the DNA incubations. O_3 was generated in an oxygen stream by UV radiation and quantified by iodine titration with $Na_2S_2O_3$ as 120 nmol/min.

2.2. *Chemical detection of singlet molecular oxygen*

9,10-Bis(2-ethylene)anthracene disulfate (EAS), kindly provided by P. Schaap (Wayne State University, Detroit), was employed to determine the amount of $^1O_2$ present in the aqueous solution. The endoperoxide of EAS as the reaction product characteristic of $^1O_2$ was separated from EAS and quantitatively determined by HPLC. Isocratic reverse-phase HPLC separation was performed on a C_{18} column using a mobile phase consisting of 70% aqueous ammonium acetate (100 mM) and 30% ethanol. The flow rate was 1.5 ml/min and detection at 229 nm.

2.3. *Transformation of* E. coli

E. coli K-12 CMK, kindly provided by Dr M. Bien, Max Planck Institut für Strahlenchemie, Mülheim was transformed by plasmid DNA pBR322 (Boehringer, Mannheim), using a $CaCl_2$ procedure as described [9]. Appropriate quantities of cells were spread on penicillin (100 μg/ml) or normal agar plates, respectively. Colonies were counted after overnight incubation at 37°C.

3. RESULTS AND DISCUSSION

A water-soluble singlet-oxygen trap, EAS, was employed to quantify the amount of $^1O_2$. Fig.1 shows the EAS endoperoxide (position 3) as the characteristic reaction product. Under conditions identical to those for the DNA incubations, its formation corresponded to 50 nmol $^1O_2$ per min in the buffer. As O atoms and O_3 might be formed in trace amounts under routine operation of the microwave discharge system, the mercury gas pressure was raised by increasing the temperature of the mercury reservoir to 45°C. No O_3-generated EAS products (see position 1 in trace C, fig.1) were then found within the detection limit in the incubation buffer (maintained at 6°C) (trace B).

Exposure of plasmid DNA pBR322 to the gas stream of $^1O_2$ generated by microwave discharge led to a decrease of transforming activity as a function of time of exposure (fig.2, table 1). Replacing

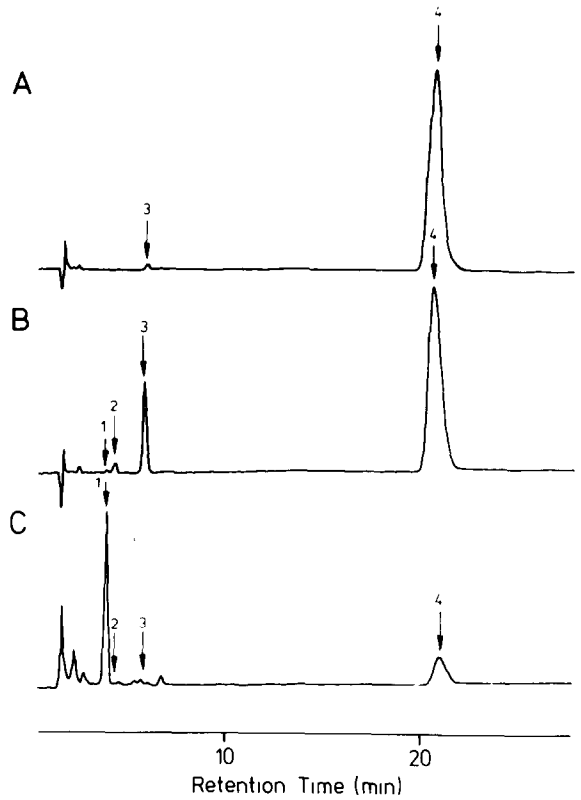

Fig.1. Chemical detection of $^1O_2$ by measuring the endoperoxide of 9,10-bis(2-ethylene)anthracene disulfate (EAS). (A) Control, EAS (1.5 mM); (B) EAS (1.5 mM) was exposed to singlet oxygen, generated by microwave discharge. (C) EAS (1.5 mM) was exposed to a gas stream containing O_3. (1) Oxidation product after reaction with O_3; (2) unknown; (3) EAS endoperoxide; (4) EAS.

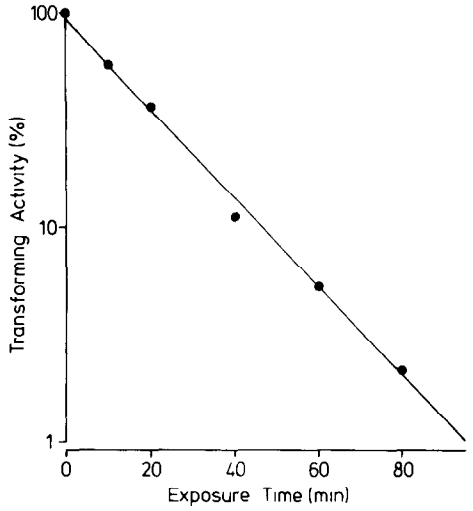

Fig.2. Transforming activity of pBR322: dependence on the exposure time of DNA solution in the microwave discharge-flow system. The reaction vessel contained 400 μl of the plasmid solution (5 μg/ml) in 5 mM Tris/0.5 mM EDTA, pH 7.5. The solution was constantly stirred by a small magnetic bar and maintained at 6°C. The amount of water lost by evaporation due to the low gas pressure was readded at 5 min time intervals. Control value: 100% corresponds to 4.9×10^{-3} transformants $\times$ (μg DNA)$^{-1}$ $\times$ (total number of cells)$^{-1}$.

water by deuterium oxide, known to increase the lifetime of $^1O_2$ more than 10-fold [10], led to a more than 2-fold increase in DNA damage assayed as transforming activity (fig.3, table 1). Further, NaN_3 as a $^1O_2$ quencher, and methionine as an alternate target for $^1O_2$ protected against DNA damage (table 1). Methionine sulfone, as oxidation product of methionine by $^1O_2$, showed only partial protection.

While there is general agreement on the oxidation of deoxyguanosine (dGuo) by $^1O_2$ as has been observed with photosensitization [11], H_2O_2/OCl^- [6], or microwave discharge [12], the present work demonstrates the capability of $^1O_2$ of destroying the biological activity of intact plasmid DNA. The loss of transforming activity is explained by base damage, most likely of guanine residues. According to Cadet et al. [7], the primary products of dGuo oxidation are unstable and undergo complex further metabolism. One mechanism leaves the purine ring system intact, whereas a second

Table 1

Effect of D_2O, NaN_3, methionine and methionine sulfone on DNA damage by singlet oxygen

	Transforming activity (%)
Control	100
$^1O_2$	32.1 ± 4.6 (9)
$^1O_2$, D_2O	14.1 ± 1.5 (5)
$^1O_2$, plus NaN_3 (20 mM)	84.3 ± 3.8 (3)
$^1O_2$, plus methionine (10 mM)	77.5 ± 1.6 (3)
$^1O_2$, plus methionine sulfone (10 mM)	58.3 ± 6.1 (3)

Data are given as means ± SE with the number of experiments in parentheses. Conditions: incubations for the transformation assay were performed as described in the legend to fig.2; incubation time 20 min

pathway leads to a cyanuric acid derivative involving the consecutive addition of two $^1O_2$. The capability of $^1O_2$ to generate single-strand breaks (SSBs), as reported by Fiel et al. [13], has been challenged [14,15] and requires further study.

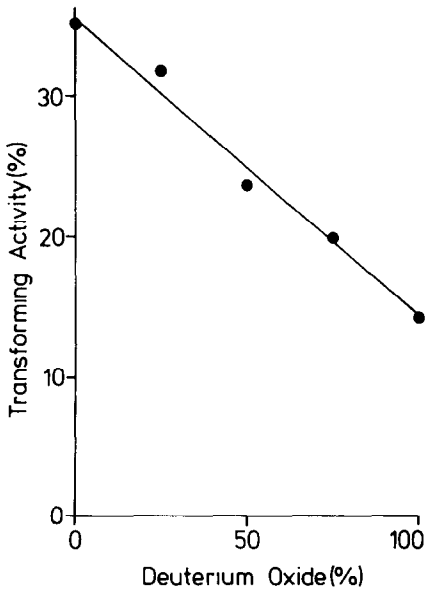

Fig.3. Dependence of transforming activity on D_2O concentration. Conditions as in fig.2, exposure time 20 min.

Recently, Lafleur et al. [16], using $^1O_2$ generated chemically from the thermodissociable endoperoxide of 3,3′-(1,4-naphthylidene)dipropionate, observed a loss of plaque-forming capacity of ϕX174 DNA. This is in agreement with the above-mentioned results. Likewise, preliminary work on the generation of $^1O_2$ by irradiation of rose bengal absorbed on silica gel beads adhered to a glass slide and measuring transforming activity of pBR322 ([17] and Schulte-Frohlinde, D. et al., unpublished) is in support of the capability of $^1O_2$ as a nonradical excited species to damage biologically active DNA.

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