Singlet Oxygen Mediated Photobinding of 8-Methoxypsoralen to DNA and Genotoxicity in *E. coli*

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Photobinding of 8-Methoxypsoralen, *E. coli* DNA Repair Test, $^1O_2$ Mediated Photobinding, 8-Methoxypsoralen

Although oxygen dependent photoreactions of 8-methoxypsoralen (8-MOP) are known, damage to DNA is mostly considered to proceed *via* photocycloaddition to this biomacromolecule. In this study the survival of colony forming ability of *E. coli* K12/343, which is deficient in DNA-repair capability, appeared to be lower in D_2O than in H_2O after exposure to the combination of 8-MOP and UV-A. Photobinding to bacterial DNA was ~40% higher in D_2O than in H_2O. However this last difference was found *only* when the bacteria were kept in the reaction medium for 1 h at 37 °C *after* the irradiation was stopped and not when they were plated out immediately afterwards. The results indicate that in this bacterial test system $^1O_2$ mediated photobinding of 8-MOP to DNA contributes to the genotoxic effect observed.

Introduction

8-Methoxypsoralen (8-MOP) is extensively used in combination with UV-A to treat skin diseases such as psoriasis, vitiligo, mycosis fungoides and lichen planus [1]. Numerous *in vitro* studies on the photochemical properties of 8-MOP indicate that both oxygen dependent type I and type II, and oxygen independent type III mechanisms may play a role in the photosensitizing effects of this compound [2]. RNA/DNA, proteins (*e.g.* enzymes) and membrane phospholipids are examples of essential biomolecules which may be damaged as a result of photoactivation of 8-MOP.

As far as photodamage to DNA is concerned most attention has been paid to a type III reaction which takes place with this biomacromolecule in particular: photocycloaddition. This can proceed in three steps: dark intercalation of 8-MOP between DNA base pairs, followed by formation of a mono-adduct and sometimes a di-adduct. Last two steps are preceded by UV-A absorption by the complex and the mono-adduct respectively. The yield of mono-adduct is very low (10^{-3}–10^{-2}) and that of the di-adduct should be still considerably lower. In this connection the question arises whether other reactive species resulting from photo-activation of 8-MOP contribute to photodamage of DNA. In this article data from experiments with *E. coli* are presented, indicating that in this bacterial test system reactions resulting from photoactivation of 8-MOP, other than the cycloaddition described above, contribute to the DNA damage observed.

Abbreviations: 8-MOP, 8-methoxypsoralen; PUVA, psoralen plus UVA; EtOH, ethanol; DMSO, dimethylsulfoxide; *E. coli, Escherichia coli;* O.C., overnight culture; PBS, phosphate buffered saline; TCA, trichloroacetic acid.

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Materials and Methods

8-[methyl-^{3}H]MOP, specific activity 81 Ci mmol^{-1}, was purchased from Amersham International plc (Amersham, U.K.) and was dissolved in EtOH (50 μCi/ml). 8-MOP as well as D_2O were obtained from Aldrich Chemical Co. Inc. (Milwaukee, U.S.A.).

As solvents DMSO, EtOH (both Merck p.a. quality) and distilled demineralized water were used.

Irradiation procedure

A Rayonet Photochemical Reactor (Southern New England UV Co.) equipped with RUL 3500 Å lamps was used. These lamps emit UV-A radiation from 320–380 nm with a maximum at ~350 nm. The light intensity was measured with a UVX radiometer equipped with a UVX-36 sensor (UV-Products, San Gabriel C.A.). The samples, in pyrex test tubes with inner diameter 1.3 cm, were irradiated in a carroussel. The temperature remained below 30 °C during the experiments.

Differential DNA-repair test with E. coli K12 strains

The colony forming ability (survival) in D_2O and H_2O of *E. coli* K12/343/753 (lac+, uvr B/rec A), which DNA repair mechanism is deficient, and *E. coli* K12/343/765 (lac−, uvr+/rec+) with an intact repair mechanism was determined in this experiment. The test was performed according to [3]; more details, *e.g.* preparation of PEPS, NRS-Agar, PBS, are found in this article. Overnight cultures (O.C.) of the bacteria strains were prepared by inocculating 30 ml PEPS-bouillon with No. 753 and 15 ml PEPS-bouillon with No. 765 and incubating overnight at 37 °C while shaking to reach the stationary growth phase. After centrifugation (Runne Zentrifugen, Heidelberg, West-Germany) at 5000 rpm of a mixture of 24 ml of No. 753 O.C. and 1.5 ml of the No. 765 O.C. the pellet was resuspended in either 6 ml D_2O-PBS or 6 ml H_2O-PBS. The titer of both bacteria in this suspension in D_2O-PBS or H_2O-PBS was determined by spreading 0.1 ml of a dilution (10^6 x) over NRS-Agar plates, in triplicate.

Samples containing 9.740 ml D_2O- or H_2O-PBS, 100 µl cellsuspension in D_2O- or H_2O-PBS and 160 µl 0.1244 mM 8-MOP solution in DMSO were put into pyrex test tubes and preincubated for 1 h at 37 °C in the dark. The tubes were irradiated for 1 h, during which, at certain time intervals, samples of 0.5 ml were taken. These were postincubated for 1 h at 37 °C in the dark. After postincubation 0.1 ml of (10^4 x) diluted samples were plated onto NRS-Agar media, in triplicate. Plates were kept at 37 °C and after 24 h the red (No. 753) and white (No. 765) colonies were counted. As a control suspensions without 8-MOP were irradiated during the same time intervals and processed in the same way.

In another experiment samples containing 4.700 ml D_2O- or H_2O-PBS, 100 µl cell suspension in D_2O- or H_2O-PBS, 20–100 µl 0.1244 mM 8-MOP solution in DMSO and 180–100 µl DMSO were put into pyrex tubes. A concentration range of 0.5; 1.0; 1.5; 2.0 and 2.5 µM 8-MOP was reached in this way. The amount of the solvent DMSO was equal in all samples. The samples were preincubated for 1 h at 37 °C in the dark, irradiated for 20 min, postincubated for 1 h at 37 °C in the dark and further processed in the same way as described above. As a control samples containing 8-MOP in the concentrations mentioned above were kept in the dark for 20 min and processed in the same way.

Binding to macromolecules of E. coli No. 753

O.C. of the bacterium strain of No. 753 was prepared by inocculating portions of 30 ml PEPS-bouillon with the bacteria and incubating overnight at 37 °C while shaking. After centrifugation at 5000 rpm for 10 min the pellets were resuspended in either 3.5 ml D_2O- or H_2O-PBS. The titer of the cell suspensions was determined by plating 0.1 ml of a dilution (10^6 x) onto NRS-Agar media, in triplicate. Pyrex test tubes were filled with 8.7 ml D_2O- or H_2O-PBS; 1.0 ml cell suspension and 0.3 ml [^{3}H]-8-MOP solution (4 mg; 0.4 µCi) and preincubated for 1 h at 37 °C in the dark. Samples of 0.5 ml were taken at certain time intervals during the 2 h irradiation. The samples were postincubated for 1 h at 37 °C in the dark. Non-covalently bound radioactivity was removed by the following procedure: 1.0 ml aqua dest. and 0.3 ml of the undiluted O.C. (to have a sufficient amount of bacterial mass to facilitate proper handling) were added to the samples after which they were submitted to dialysis for 3 days at 15 °C against a bulk volume of water which was regularly refreshed (Cellulose tubing Visking No. 16). After dialysis the samples were centrifuged at 5000 rpm for 20 min. To separate DNA/RNA from protein a modification of the method of [4] was followed: The pellets were extracted with 3 ml 5% TCA at 100 °C for 15 min and centrifuged at 5000 rpm for 30 min. The supernatans, containing DNA/RNA, was collected and the remaining pellet, containing protein, was dissolved in 3 ml 0.1 N NaOH. The radioactivity of the DNA/RNA and the protein fraction was determined by mixing 1 ml of the fractions with 10 ml Opti-phase and counting with a Packard Tri-Carb 4640 liquid scintillation meter. The amount of protein and DNA/RNA in both fractions was determined according to

[5] and to [6] respectively. The DNA/RNA fraction appeared to contain a small amount of protein. However because the protein fraction was free of DNA/RNA the amount of 8-MOP irreversively bound to each of both macromolecules could be determined.

Results and Discussion

In general photomutagenic effects are attributed to the formation of mono-adducts and di-adducts of a furocoumarin with DNA. Di-adducts leading to cross-linking of DNA-strands induces mutagenicity at a higher frequency than mono-adducts [7]. In earlier work [8] we found indications for the involvement of oxygen dependent reactions in the induction of mutations by 8-MOP in a repair deficient bacterial strain (*E. coli* K-12/343/113 uvr B). More recently [9] we tried to correlate the induction of genotoxic effects in this strain with photobinding of 8-MOP to the *E. coli* bacterial macromolecules *in situ*. In this test we assayed the colony forming ability (survival) of the cells and the induction of arg^+ back mutations [9]. In D_2O mutagenicity appeared to be increased with ~80% with respect to that in H_2O, what was still somewhat underestimated if the ~25% lower survival in D_2O was taken into account. Because the lifetime of $^1O_2$ is estimated to be 14–18 times longer in D_2O than in H_2O [10] and that of triplet excited 8-MOP is only slightly affected (D_2O: $k \sim 1.87 \times 10^5$ and H_2O: $k \sim 1.63 \times 10^5\ s^{-1}$; [9]) the conclusion seems attractive that $^1O_2$ mediated processes account for the increased mutagenicity.

To get further support for this conclusion we did in the present study some experiments with another *E. coli* test system for determining genetic damage: a DNA-repair test. In this test [3] two derivatives of strain 343/113 of *E. coli* K-12 were used which have the same physicochemical constitution; they *only* differ from each other in that No. 765 can repair certain DNA-damage (uvr B, rec A) and No. 753 not. Genotoxic stress to be studied, is applied to such an extent that No. 765 completely survives. The essential property of this test is then that a *difference* in survival of colony forming ability between both strains, as a result of stress *e.g.* exposure to 8-MOP + UV-A, is caused by *direct* damage to DNA and not *indirectly*, by *e.g.* reaction with proteins (enzymes) involved in DNA repair. (The two *E. coli* strains also differ in their auxotrophic requirements, No. 765 is lac– and No. 753 lac+, and therefore their individual survival can be determined in mixtures in which both bacteria are present. This is done by plating onto the same agar medium, with neutral red as a pH indicator, on which the lac+ appears as red colonies and lac– as white.)

As can be seen in Fig. 1 and 2 there is a difference in survival of colony forming ability of No. 753, exposed to 8-MOP + UV-A in D_2O and in H_2O. (No decrease of survival was found with No. 765 exposed to 8-MOP + UV-A, neither was this the case with No. 753 exposed to either 8-MOP or UV-A alone). These results correspond to those already found by [9] namely an increased genotoxicity in D_2O compared to H_2O.

With respect to the photobinding experiments it is important to notice that the strains No. 765 and No. 753 have the same physiochemical constitution as the *E. coli* we previously used [9] as well as that of

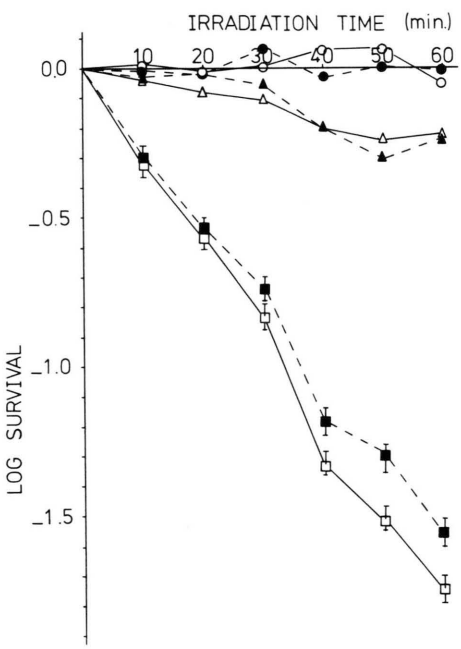

Fig. 1. Colony forming ability (survival), as a function of irradiation time, of 2 strains of *E. coli* K12/343 differing in DNA-repair capability. Repair proficient No. 765: in the presence of 8-MOP in H_2O-medium (----●----) and in D_2O-medium (——○——). Repair deficient No. 753 (uvr B, rec A) in the absence of 8-MOP in H_2O-medium (----▲----) and in D_2O-medium (——△——); in the presence of 8-MOP in H_2O-medium (----■----) and in D_2O-medium (——□——). [8-MOP] = 2.0 mm; cell titer = 1.5×10^8 viable cells/ml; UV-A 320–380 nm, max = 350 nm, I at the tube ~0.04 $mJs^{-1}\ cm^{-2}$. The bars represent the standard deviation of 3 independent experiments.

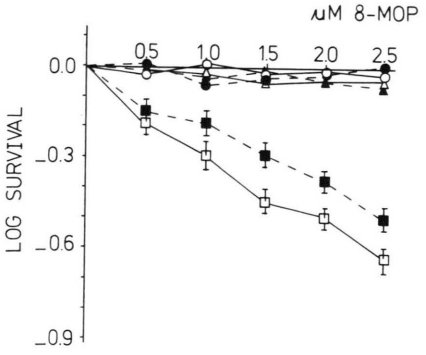

Fig. 2. Colony forming ability (survival), as a function of 8-MOP concentration, of 2 strains of *E. coli* K 12/343 differing in DNA-repair capability. Repair proficient No. 765: after exposure to UV-A in H_2O-medium (----●----) and in D_2O-medium (——○——). Repair deficient No. 753 (uvr B, rec A) without irradiation in H_2O-medium (----▲----) and in D_2O-medium (——△——); after exposure to UV-A in H_2O-medium (----■----) and in D_2O-medium (——□——). Cell titer = 1.5×10^8 viable cells/ml. UV-A, irradiation time = 20 min. See also Fig. 1.

the original *E. coli* wild type. Without post-incubation covalent binding to protein appeared to be increased with ~15% whereas that to DNA/RNA did not change at all when D_2O instead of H_2O was used as a medium [9]. As can be seen in Fig. 3 post-incubation for 1 h at 37 °C alters these figures: both irreversible binding to protein and to DNA/RNA increases going from H_2O to D_2O. That the difference between D_2O and H_2O with respect to covalent binding to protein is larger with post-incubation (~40%) than without (~15%) supports a mechanism according to [11] and [12]: Instable products from the reaction of 8-MOP with $^1O_2$ generated by the furocoumarin itself (see also [13]) irreversibly bind to protein. (Although it seems quite plausible that this $^1O_2$ mediated irreversible binding to protein contributes to the overall genotoxic effect of the 8-MOP + UV-A treatment (see also [14]) this does not explain the difference in survival between No. 765 and No. 753 (Fig. 1 and 2). As No. 765 completely survived under the experimental conditions applied the genotoxic effect observed with No. 753 is caused by *direct* damage to DNA only. This merely is the consequence of the characteristic property of this DNA-repair test system. ([3]) A difference between D_2O and H_2O with regard to photobinding of 8-MOP to DNA was not found *without* incubating the suspension after UV-A exposure [9] but only with a post-incubation of 1 h (~40%, see Fig. 3). Because of the short life time of the 8-MOP triplet state the *increased* covalent binding to DNA/RNA can not result from "classical" photocycloaddition of 8-MOP to DNA. A mechanism comparable to that underlying irreversible binding to protein in the presence of oxygen [11] and [12] seems more attractive: *i.c.* reactive photoproducts formed by reaction of 8-MOP with singlet

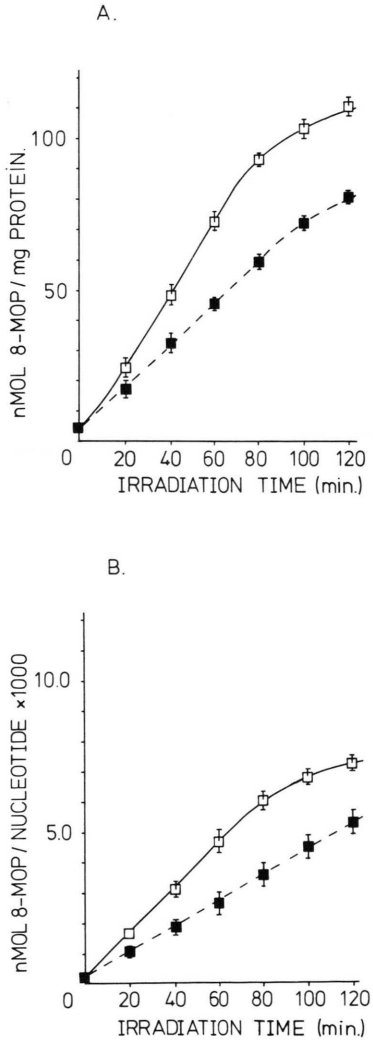

Fig. 3. Photobinding in D_2O (——□——) and in H_2O (----■----) of 8-MOP to macromolecules of *E. coli* K 12/343/753/uvr B, rec A in saline phosphate buffer pH = 7.0. Cell titer = 3×10^9 viable cells/ml; [8-MOP] = 0.18 mM; UV-A: 320–380 nm, max = 350 nm, I at the tube ~2.5 $mJs^{-1}cm^{-2}$. The bars represent the standard deviation of 3 independent experiments. Binding to proteins (panel A) and to DNA (panel B).

oxygen generated by the excited furocoumarin itself, irreversibly bind to DNA. The increase (40%) of covalent binding to DNA going from H_2O to D_2O indicates that in this bacterial test system $^1O_2$ production contributes to the damage to DNA resulting from exposure to the combination of 8-MOP + UV-A.

Singlet oxygen produced by the excited 8-MOP may damage biomacromolecules directly, or indirectly *e.g. via* oxidation of 8-MOP as described above. In this connection it is of interest that [15] found that furocoumarins can produce $^1O_2$ even when they are complexed with or covalently bound to DNA.

Whether damage to DNA by these $^1O_2$ mediated processes is more easy repaired and thus less mutagenic than that caused by photocycloaddition of 8-MOP is an open question. However an experimental system to investigate this will not be easy found, not in the last place because of the fact that damage to bio(macro)molecules other than DNA can contribute to the eventual mutagenic effect.

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