EXPERIMENTAL EVIDENCE FOR COVALENT BINDING OF AN ORGANIC NITROXIDE FREE RADICAL TO RADIATION INDUCED DNA RADICALS

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1. Introduction

Organic nitroxide free radicals have recently received great interest due to their radiosensitizing ability under anoxic conditions [1,2]. At low concentrations, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TAN) is found to be less toxic and a more effective radiosensitizer than other nitroxide free radicals studied [2]. TAN may also be more effective than O_2 in enhancing radiation induced damage to DNA [3], although O_2 generally is more effective than TAN in enhancing radiation induced lethality to bacteria [2,4].

The present study was undertaken to throw light on the radiation induced interaction of TAN with DNA in dilute neutral aqueous solutions. Pulse radiolysis investigations have revealed that OH induced DNA transients interact rapidly with TAN [5,6]. Under our experimental conditions, TAN was found to be bound covalently to DNA when present during irradiation of anoxic solutions. The results indicate that OH induced DNA radicals may form stable covalent bonds with TAN, whereas e^-_{aq} induced DNA radicals lack this ability.

2. Materials and methods

2.1. *Chemicals*

Calf thymus DNA (highly polymerized sodium salt) was purchased from Sigma Chemical Company and used without further purification. TAN was synthesized from 2,2,6,6-tetramethyl-4-piperidone-HCl (Aldrich Chemical Company) according to published procedures [7] with some minor modifications.

^{3}H-TAN was synthesized from ^{3}H-2,2,6,6-tetramethyl-4-piperidone (The Radiochemical Center, Amersham). ^{3}H-TAN was obtained with specific activity of about 1.4 mCi/mmole and was recrystallized prior to use.

2.2. *Irradiation conditions*

Solutions of TAN (10^{-5}–10^{-4} M) and DNA (0.25 –2 mg/ml) in 0.01 M NaCl were irradiated with electrons from a 4 meV linear accelerator, with an average dose rate of about 10^4 rad/sec.

The solutions were flushed with either O_2, N_2 or N_2O prior to and during irradiation.

2.3. *Chromatography*

Test for covalent bonds between TAN and DNA was performed on 1 ml irradiated samples added to the top of a 0.6 × 100 cm Sephadex G-25 column, and eluted with 0.01 M NaCl. The extent of binding of TAN to DNA was determined from the fraction of the total tritium activity found in the front-running DNA peak.

The concentration of DNA and of TAN in the eluate was determined from absorbance measurements at 260 nm and at 235 nm, respectively, using a Zeiss PMQ II spectrophotometer.

Tritium activity was measured with a liquid scintillation counter (Beckman LS-150) with PPO (5 g) - naphthalene (100 g) - dioxane (1000 ml) as scintillator solution.

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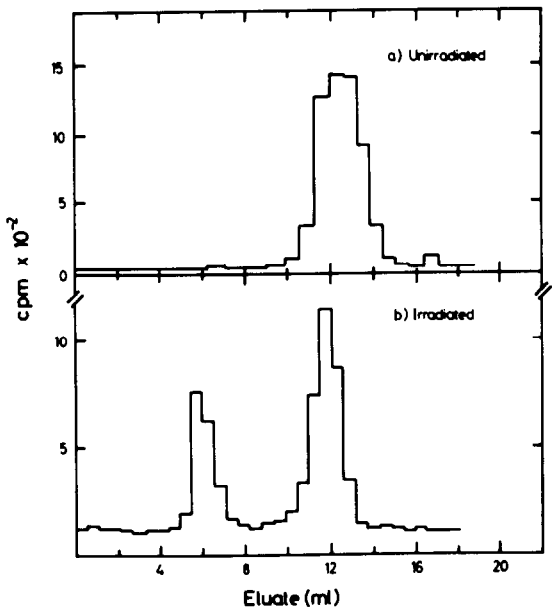

Fig. 1. Radiation induced binding of ^{3}H-TAN to DNA in solutions equilibrated with N_2. Aliquots (1 ml) of (a) unirradiated and (b) irradiated solutions (50 krad) of DNA (1 mg/ml) and TAN (1×10^{-4}M) were added on top of a Sephadex G-25-column. The latter was eluted with 0.01 M NaCl and the ^{3}H-activity profile of the eluate was recorded.

3. Results and discussion

ESR measurements showed that unirradiated TAN in anoxic solution at room temperature remained stable over periods of time longer than the duration of the present experiments. A possible reason for the pronounced stability of unirradiated TAN is that its free radical, located at the nitrogen-oxygen bond, is sterically hindered through the presence of the neighbouring methyl groups [8].

When solutions containing TAN and DNA were chromatographed on Sephadex G-25 columns, spectrophotometric mesurements of the eluate revealed two well separated peaks, the first one due to DNA, the second one due to TAN.

When a solution of DNA was irradiated alone and under anoxic conditions, and a few minutes after irradiation mixed with TAN, no ^{3}H-activity was present in the DNA peak of the eluate. Similarly if TAN was irradiated, and then mixed with DNA, no reaction occurred.

Typical examples of the ^{3}H-activity profile in the eluate for unirradiated samples and for samples irradiated under anoxic conditions are presented in fig. 1. Only in the latter cases was ^{3}H-activity found in the DNA peak. This activity was eluted as a single peak together with DNA when run through a second column.

We conclude from these observations that radiation induced binding of TAN to DNA must be due to formation of covalent bonds between either

(1) DNA transients and TAN,

(2) DNA transients and TAN transients, or

(3) TAN transients and DNA.

Preliminary experimental results indicate that binding of TAN to DNA is due mainly to interaction (1) above. Thus, under certain experimental conditions (i.e. in presence of N_2O), the number of molecules of TAN bound per 100 eV (G-value) was found to increase linearly with the ratio $(DNA)_o/(TAN)_o$. Experimental proof that covalent binding of TAN to DNA may result from the interaction of TAN with long-lived transients of DNA has recently been obtained by rapid mixing technique [9].

DNA transients may be formed either by interaction of DNA with radiation induced water radicals (e^-_{aq}, OH and H), or by direct radiation effect on DNA. The latter effect can be ignored for the low concentrations of DNA used in the present experiments. In irradiated neutral water solutions e^-_{aq} and OH are formed in approximately equal yields, both of which are much higher than that of H [10]. For sufficiently low values of the ratio $(TAN)_o/(DNA)_o$, DNA may be assumed to scavenge essentially all radicals induced in water, and the following reactions involving TAN should be considered:

$$\text{TAN} + \text{DNA-OH}^\circ \rightarrow \text{TAN}_{\text{bound}} \quad (1)$$

$$\text{TAN} + \text{DNA-}e^- \rightarrow \text{TAN}_{\text{bound}} \quad (2)$$

$$\text{TAN} + \text{DNA-H}^\circ \rightarrow \text{TAN}_{\text{bound}} \quad (3)$$

$$\text{TAN} + \text{DNA-OH}^\circ \rightarrow \text{TAN}_{\text{red}} + \text{DNA}_{\text{ox}} \quad (4)$$

$$\text{TAN} + \text{DNA-}e^- \rightarrow \text{TAN}_{\text{red}} + \text{DNA} \quad (5)$$

$$\text{TAN} + \text{DNA-H}^\circ \rightarrow \text{TAN}_{\text{red}} + \text{DNA} \quad (6)$$

A detailed study of reactions (1)–(6) is outside the scope of the present work. However, we obtained

Table 1
Radiation induced covalent binding of TAN to DNA in 0.01 M NaCl, pH 7.0, for a dose of 10 krad.

$(DNA)_o \times 10^3$ [a]	$(TAN)_c \times 10^5$	$\frac{(^3H)_{DNA}}{(^3H)_{DNA} + (^3H)_{TAN\text{-}H}}$ [d]		
		N_2	N_2O	O_2
3	1	0.46	0.74	0.002
3	2	0.47[b]	0.71[c]	–
3	4	–	0.72	–
6	2	–	0.66	–

[a] Calculated concentration of nucleotides, assuming an average molecular weight of 330.
[b] and [c] Mean value of 3 respectively 5 experiments.
[d] Low molecular TAN-product unable to interact with DNA radicals.

information regarding their relative importance by performing experiments in N_2 and N_2O saturated solutions with $(DNA\text{-nucleotides})_o > 3 \times 10^{-3}$ M and $(TAN)_o \leqslant 4 \times 10^{-5}$ M. A radiation dose of 10 krad was assumed to deplete TAN through interactions with DNA radicals. Thus, binding of TAN to DNA remained constant when the dose was increased from 6 to 20 krad.

Under the above conditions the value of the radio $(TAN_{bound})/[(TAN_{bound}) + (TAN_{red})]$, (hereafter denoted β), is equal to that of the ratio $(^3H)_{DNA}/[(^3H)_{DNA} + (^3H)_{TAN\text{-}H}]$ (denoted β_{exp}), as determined experimentally.

Values for β_{exp} are given in table 1. They are, for a dose of 10 krad and a particular gas, independent of $(DNA)_c$ and $(TAN)_o$ for the concentration range studied. Hence the two assumptions made above, viz. that DNA scavenges all radicals induced in water, and that TAN is completely depleted in interactions with DNA radicals, seem to be justified.

The data in table 1 further show that the values for β_{exp} are lower in N_2 than in N_2O. The latter compound is an effective electron scavenger [10]. Since e^-_{aq} may be transformed into OH radicals by N_2O [10], reactions (1) and (5) are probably dominating. If TAN is only bound to OH° induced DNA radicals, and if TAN interacts with all DNA radicals at diffusion controlled rates, the value for β is given by:

$$\beta = \frac{G_{OH}}{G_{OH} + G_{e^-} + G_H}, \qquad (7)$$

where G_{OH}, G_{e^-} and G_H are the numbers of the various radical species formed per 100 eV of energy absorbed.

Using the values $G_{OH} = 2.8$, $G_{e^-} = 2.8$, and $G_H = 0.5$ [11] and assuming that N_2O converts all e^-_{aq} into OH radicals, the values for β are 0.46 and 0.92 with N_2 and N_2O, respectively. We see that the former is in excellent agreement with the values for β_{exp} for N_2 as listed in table 1. For N_2O saturated solutions, we obtained an average value of 0.71 which is substantially lower than the theoretical value of 0.92. This discrepancy may be due to (a) competition between DNA and N_2O for e^-_{aq}, (b) occurrence of reaction (4), or (c) interactions between OH°-induced DNA radicals when these are present at higher concentrations.

When experiments were performed on oxygen saturated solutions, (see table 1) no binding of TAN to DNA was observed, indicating that oxygen competes effectively with TAN for radiation induced DNA-transients. An alternative factor, which may be of importance, is that oxygen under certain conditions quenches the ESR signal of TAN [12].

The result of the present experiments suggests that (1) a large fraction of radiation induced DNA radicals interact with TAN under anoxic conditions, and (2) TAN is covalently bound to OH° induced DNA radicals. The latter effect may be essential for the radiosensitizing effect of TAN in biological systems.

Tritiated TAN may now be used to characterize OH° induced radical sites within DNA and other vital

molecules, and to study the radiosensitizing effect of organic nitroxide free radicals in chemical and biological systems.

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