Indirect EPR Evidence for the Production of Singlet Oxygen in the Photosensitization of Nucleic Acid Constituents by Proflavine

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EPR spectroscopy is used to analyse the influence of D_2O and NaN_3 on the free radicals induced in frozen aqueous solutions of 5'-thymidine monophosphate photosensitized ($\lambda > 320$ nm) by proflavine at 205 K. The results can be explained if the production of singlet oxygen during the process is assumed.

Peroxides free radicals can be induced in DNA components by proflavine and visible light ($\lambda > 320$ nm) according to the following scheme

$$RH_2^{\cdot} + O_2 \rightarrow RH_2 - OO^{\cdot} .$$

$RH_2^{\cdot}$ being a radical species resulting from a reaction between the substrate RH and electrons liberated by biphotonic ionization of the chromophore[1]. When the photosensitization is realized at 205 K, no peroxide radical is observed and the total amount of paramagnetic centers ($RH_2^{\cdot}$) is less in presence than in absence of oxygen. This difference is especially important in the case of pyrimidine nucleotides and particularly of 5'-thymidine monophosphate (5'-dTMP). The proposed interpretation involves an energy transfer from the dye triplet state toward an excited singlet state of oxygen[1].

To test the production of singlet oxygen molecules, we have analysed the modifications induced in the yield or in the nature of free radicals by introducing agents known to influence the lifetime of singlet oxygen. Among them, azide ion (N_3^-) is known to quench that excited state[2] and D_2O enhances the corresponding lifetime[3].

In this short note we described the results of experiments performed with 5'-dTMP as substrate, using electron paramagnetic resonance (EPR) spectroscopy.

The EPR spectra are recorded at 77 K with an E-3 Varian spectrometer. A Varian Spectrosystem 100 is used for the determination of the corresponding number of free radicals. The aqueous solutions containing 5'-dTMP (Calbiochem 10^{-1} M) and proflavine (British Drug Houses 5×10^{-4} M) are introduced in quartz tubes of 4 mm diameter, rapidly frozen in liquid nitrogen. Throughout the irradiation period (one hour exposure to an Osram HBO 500 W high pressure mercury vapour lamp), the samples are dipping in a nitrogen beam maintained at 205 K using a Varian accessory temperature. All radiation below 320 nm was eliminated by a Jenaer Glasswerk Schott WG 345 filter. The influence of oxygen is studied by comparing the results obtained either in N_2 or in O_2 saturated solutions.

First of all, it must be noted that the addition of NaN_3 (Merck – 10^{-2} to 10^{-1} M) to the proflavine-nucleotide solutions does not modify any characteristic of the dye triplet state, detected during direct irradiation in the cavity at 77 K. On the contrary, in D_2O (99.75% – Merck) the proflavine-T_1 life-time is substantially increased: 2.31 ± 0.05 sec as compared to 1.8 ± 0.02 sec in H_2O. An increase of about 25% is observed in the corresponding population n_{T_1}. At 205 K, the visible irradiation of proflavine-NaN_3 mixtures does not give any free radical, even if oxygen is present. On the other hand, in photosensitized frozen aqueous solutions of 5'-dTMP, H-adduct radicals are the essential paramagnetic centers observed. As noted in Table I, NaN_3 has no influence on the amount and on the type of free radicals when it is added to N_2 saturated solutions before freezing. But, in presence of O_2, the addition of NaN_3 leads to the appearance of peroxide radicals whose number increases with the concentration in singlet oxygen quencher. When D_2O replaces H_2O as the solvent, the free radicals mainly induced are $RHD^{\cdot}$, independently of oxygen presence. The analysis of the quantitative results shows that, without oxygen, the

Table I. Relative EPR intensities * and types of main free radicals detected at 77 K after photosensitization of 5'-dTMP at 205 K.

		N_2 Saturated solutions		O_2 Saturated solutions	
Solvent	Addition of NaN_3 [10^{-2} M]	Yield [arb. units]	Type of free radicals	Yield [arb. units]	Type of free radicals
H_2O	0	25	$RH_2^{\cdot}$	6	$RH_2^{\cdot}$
	1.25	23.8	$RH_2^{\cdot}$	2.2	$ROO^{\cdot}$
	2.5	24.5	$RH_2^{\cdot}$	2.5	$ROO^{\cdot}$
	5	26	$RH_2^{\cdot}$	2.7	$ROO^{\cdot}$
	10	23.2	$RH_2^{\cdot}$	4.8	$ROO^{\cdot}$
D_2O	0	27	$RHD^{\cdot}$	5.3	$RHD^{\cdot}$

* These values were obtained by averaging the results of three series of experiments within a reproductibility not exceeding 15%.

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total amount of free radicals is higher in heavy water than in H_2O, in complete agreement with the corresponding increase observed in the dye triplet state characteristics. Finally, the differences observed in the yields between N_2 and O_2 saturated D_2O solutions are parallel to those revealed by the comparison of the two corresponding H_2O solutions.

At 205 K, the irradiation with $\lambda = 440$ nm does not give any radical even if oxygen is present. If singlet oxygen molecules are produced by energy transfer from the triplet state of the chromophore, this result indicates that under our experimental conditions, these excited oxygen molecules are not able to give peroxide radicals.

On the other hand, NaN_3 has only an influence when oxygen is present. Even if the quenching properties of this salt are not demonstrated in frozen aqueous solutions, until now, the present results cannot be interpretated without admitting that it is these properties which govern this behavior of azide ion. Under these conditions, 5′-dTMP being a very poor singlet oxygen acceptor, the competitive reaction between this nucleotide and NaN_3 to capture singlet oxygen molecules would be in favor of the latter which could then restore ground-state triplet state oxygen and lead to peroxide radicals. These observations could thus be considered as indirect proofs of a singlet oxygen production during the photosensitization by proflavine.

At room temperature, the occurence of a singlet oxygen mechanism has effectively been proven in photodynamic oxidations of guanosine by a variety of sensitizers [5]. The present conclusions indicate that such a mechanism would also be involved in the photooxidation of other DNA components sensitized by proflavine.

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