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Kinetic behavior of the reaction between hydroxyl radical and the SV40 minichromosome

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Abstract

Aqueous solutions containing the minichromosomal form of the virus SV40 and the radical scavenger DMSO were subjected to gamma-irradiation, and the resulting formation of single strand breaks (SSB) was quantified. Under the irradiation conditions, most SSBs were produced as a consequence of hydroxyl radical ($\cdot\text{OH}$) reactions. By controlling the competition between DMSO and the viral DNA substrate for $\cdot\text{OH}$, we are able to estimate the rate coefficient for the reaction of $\cdot\text{OH}$ with the SV40 minichromosome. The results cannot be described adequately by homogeneous competition kinetics, but it is possible to describe the rate coefficient for the reaction as a function of the scavenging capacity of the solution. The experimentally determined rate coefficient lies in the range $1 \times 10^9 - 2 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ at 10^7 s^{-1} , and increases with increasing scavenging capacity.

1. Introduction

The indirect effect of ionizing radiation plays a significant role in the irradiation of biological systems (Chapman 2003). The irradiation of cells in the presence of various radical scavengers has shown that cell killing is strongly correlated with one product of water radiolysis, the hydroxyl radical ($\cdot\text{OH}$) (Roots and Okada 1975, Dahm-Daphi et al. 2000). Particular emphasis is placed on the importance of the reaction of this species with DNA (Bernhard and Close 2003). Therefore we have been investigating the reactivity of $\cdot\text{OH}$ with DNA in model systems. One parameter commonly used to characterize the chemical reactivity of one species with another is the second-order rate coefficient or rate constant (Moore 1981).

The hydroxyl radical is a very reactive species. It reacts at near diffusion controlled rates with a wide range of compounds (Buxton et al. 1988), including various forms of DNA (Milligan et al. 1996). DNA is however a macromolecule. Reactions between two species, one of which is a macromolecule, that take place at or near the diffusion controlled limit are typically poorly described by regular homogeneous chemical kinetics (Schwartz 1971; Mark et al. 1989, Grima and Schnell 2006). The second-order rate coefficient for the reaction is found empirically to be a function of the lifetime of the faster diffusing species (Udovicic et al. 1991), and it is sometimes seen described as a time-dependent rate coefficient. The effect

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can be observed with $\cdot\text{OH}$ by irradiating solutions containing various amounts of added radical scavengers, because the lifetime of $\cdot\text{OH}$ depends on the scavenger concentration.

We have previously determined the rate coefficient for the reaction between $\cdot\text{OH}$ and DNA in the form of several plasmids, using single-strand break (SSB) formation as a means of following the reaction (Milligan et al. 1996). This is a convenient method because of its high sensitivity and only modest (microgram) amounts of the plasmid are required. Plasmids are supercoiled closed-circular double-stranded DNA molecules of a few thousand base pairs in length, having molecular weights of a few million Daltons (Watson et al. 2006). To provide a better model system for chromatin, we have most recently used as a DNA substrate the minichromosomal form of the mammalian virus SV40. The SV40 minichromosome has found frequent use as a model for DNA in cellular chromatin (van Holde 1988, Riedinger et al. 2002). It contains a closed-circular double-stranded DNA molecule 5243 bp in length associated with an approximately equal mass of histone proteins (Blasquez et al. 1988). The DNA and histones are organized into 20 – 24 nucleosomal units. Each nucleosome consists of 146 bp of double-stranded DNA wrapped around a central histone octamer to form 1.8 turns of a left-handed superhelix with 80 bp per turn and a pitch (distance between adjacent turns) of 2.7 nm (Saenger 1988). Each nucleosome assumes an approximately cylindrical shape about 11 nm in diameter and 6 nm in height. Short stretches (40 – 60 bp) of linker DNA exist between nucleosomes, such that the average repeat length in SV40 is 180 – 200 bp, although the overall location of nucleosomes in SV40 is neither random nor unique (van Holde 1988).

Here we report values for the rate coefficient for the reaction between $\cdot\text{OH}$ and polynucleosomal DNA in the form of the SV40 minichromosome, and compare them with the values previously determined using plasmid DNA (Milligan et al. 1996) and also single stranded calf thymus DNA (Udovicic et al. 1991).

2. Materials and methods

2.1. Preparation of DNA substrate

The preparation and purification of the SV40 minichromosome (5243 bp, $3.4 \times 10^6 \text{ g mol}^{-1}$ DNA) has been described previously (Oudet et al. 1989; Milligan et al. 1993). It was isolated from the nuclei of infected CV-1 cells late in the infection cycle and purified by sedimentation through sucrose density gradients. Residual low molecular weight contaminants were removed by extensive dialysis. DNA concentrations were determined spectrophotometrically at $\lambda = 260 \text{ nm}$. The presence of histone proteins was verified by polyacrylamide gel electrophoresis in the presence of SDS.

2.2. Irradiation

Solutions contained the minichromosomal form of SV40 ($12 - 200 \mu\text{g mL}^{-1}$), DMSO ($3 \times 10^{-4} - 1 \times 10^{-2} \text{ mol L}^{-1}$), 0.13 mol L^{-1} sodium chloride, and $10^{-2} \text{ mol L}^{-1}$ sodium phosphate (at pH 7.0). The solutions were irradiated in a AECL GammaCell-1000 isotopic instrument (^{137}Cs γ -rays, 662 keV) under aerobic conditions with in 0.5 or 1.5 mL polypropylene tubes at 25°C . Depending on the DMSO concentration, the maximum dose was 40 – 400 Gy, and the dose rate $4.1 \times 10^{-2} - 0.25 \text{ Gy s}^{-1}$. Dose rates were determined with the Fricke system (Spinks and Woods 1990).

2.3. Quantifying strand break yields

After irradiation, the SV40 minichromosome was assayed for strand breaks by using agarose gel electrophoresis. To do so it was necessary to remove the histone proteins by SDS treatment after irradiation but prior to electrophoresis. The procedure has been described

previously (Milligan et al. 1993). Briefly, the D_0 radiation dose is that required to decrease the fraction of the supercoiled (SSB free) form of the SV40 DNA by a factor of e . If SSBs are assumed to adhere to approximate a Poisson distribution, a decrease by a factor of e in the SSB free fraction is equivalent to a mean of one SSB per SV40. The value of D_0 is equal to the reciprocal of the slope m of a straight line fitted to a semi logarithmic yield dose plot. At the D_0 dose, the concentration of the SSB product is equal to the concentration of the SV40 viral genome. The G -value for SSB formation is equal to the quotient of this concentration and the D_0 dose.

3. Results

The results of a typical experiment are reproduced in Figure 1. In this case, the SV40 minichromosome at a DNA concentration of $30 \mu\text{g mL}^{-1}$ was irradiated in the presence of $5 \times 10^{-3} \text{ mol L}^{-1}$ DMSO. For each radiation dose, the fraction of intact supercoiled SV40 DNA remaining was determined by using agarose gel electrophoresis. Under these conditions, the supercoiled form of SV40 (which is free of SSBs) decreases exponentially from a mole fraction of about 0.9 in the absence of any irradiation to about 0.2 for a dose of 240 Gy. The SSB radiation chemical yield or G -value (denoted by $G(\text{SSB})$) under these conditions can be calculated from the slope of the straight line fitted to the data in Figure 1.

The experiment whose results are shown in Figure 1 was repeated with six different concentrations of SV40. Seven to ten different radiation doses were generally used for the determination of $G(\text{SSB})$ at each SV40 concentration. The SSB yield increases from about 2×10^{-5} to about $2 \times 10^{-4} \mu\text{mol J}^{-1}$ as the concentration of SV40 DNA increases from 12 to $100 \mu\text{g mL}^{-1}$ and the DMSO concentration is held constant at $5 \times 10^{-3} \text{ mol L}^{-1}$. In the form of figure 2, which is a double reciprocal plot, the value of $1/G(\text{SSB})$ is proportional to the value of $1/[\text{SV40}]$. Please note that the DNA concentration has been expressed as the concentration of nucleotide residues. This calculation assumes a molecular weight of 325 g mol^{-1} per nucleotide residue. The agreement of the fitted straight line with the data suggests that homogeneous competition kinetics can be applied to SSB formation under these conditions (see Discussion).

From the slope and intercept of the straight line in figure 2, it is possible to calculate the rate coefficient for the reaction of $\cdot\text{OH}$ with the SV40 minichromosome (k_2 , see Discussion). Similar plots can be constructed, each one containing data collected at a different but constant concentration of DMSO. Using this method, we have determined the value of k_2 at five different DMSO concentrations (not shown).

Figure 3 shows these values of k_2 plotted as a function of the DMSO concentration. The DMSO concentration has been recalculated as the $\cdot\text{OH}$ scavenging capacity ($\sigma(\cdot\text{OH})$), defined as the product of the DMSO concentration and the second-order rate constant for its reaction with $\cdot\text{OH}$. This makes it possible to compare our results with those obtained using other scavengers. The value of k_2 increases from *ca.* 1×10^9 to *ca.* $1 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ (10 fold) as $\sigma(\cdot\text{OH})$ increases from *ca.* 2×10^6 to *ca.* $5 \times 10^8 \text{ s}^{-1}$ (250 fold). Trends of similar direction and magnitude have been reported previously for single stranded calf thymus DNA (Udovicic et al. 1991) and for double stranded plasmid DNA (Milligan et al. 1996). Both of these are also plotted in figure 3.

4. Discussion

Radiolysis of water produces $\cdot\text{OH}$ radicals (Spinks and Woods 1990). Of those that escape the spurs, the majority are scavenged by DMSO (reaction 1) while only a small minority react with the DNA substrate (reaction 2).

The rate coefficients for these reactions are k_1 and k_2 respectively. The only product of reaction 2 that we detect is a SSB in the DNA component of the SV40 minichromosome. Under some conditions, the products of reaction 1 (methyl and methylperoxyl radicals) are reactive with DNA. The methyl radical is reactive with DNA only under anoxic conditions where it has a long lifetime (Milligan and Ward 1994). Under aerobic conditions such as we have employed here, the methyl radical is rapidly scavenged by oxygen to form the methylperoxyl radical (Marchaj et al. 1991). This species is a weak single electron oxidizing agent with $E_7(\text{ROO}^\bullet, \text{H}^+ / \text{ROOH}) = +1.06 \text{ V}$ (Merenyi et al. 1994). Guanine bases ($E_7(\text{G}(-\text{H})^\bullet, \text{H}^+ / \text{G}) = +1.29 \text{ V}$, Steenken and Jovanovic 1997) are the most easily oxidized sites in DNA (Colson et al. 1992), but the methylperoxyl radical is able to oxidize them only to a minor extent (Milligan et al. 1996). Since oxidation products of guanine bases do not produce SSBs under the conditions we have used here (Cullis et al. 1996), this reaction may be safely ignored. Redox active amino acids present in the histone proteins of the SV40 minichromosome such as tyrosine ($E_7 = +0.93 \text{ V}$, Harriman 1987; DeFilippis et al. 1989) and tryptophan ($E_7 = +1.03 \text{ V}$, Harriman 1987; Wardman 1989) may be oxidized more extensively by the methylperoxyl radical. However the resulting amino acid radical species do not produce strand breaks in DNA (Milligan et al. 2004).

Therefore the role of reaction 1 is to irreversibly scavenge $\bullet\text{OH}$ and prevent their reaction with the SV40 minichromosome. The competition between these two reactions can then be quantified by the usual application of homogeneous competition kinetics (equation 1).

$$\frac{1}{G(\text{SSB})} = \frac{1}{G_0(\text{SSB})} + \frac{k_1[\text{DMSO}]}{G_0(\text{SSB})k_2[\text{SV40}]} \quad (1)$$

In equation 1, $G(\text{SSB})$ and $G_0(\text{SSB})$ represent the SSB yield in the presence and absence respectively of DMSO.

If equation 1 is an accurate description of the competition between DMSO and the SV40 minichromosome for $\bullet\text{OH}$, then the relationship between $1/G(\text{SSB})$ and either $1/[\text{SV40}]$ or $[\text{DMSO}]$ should be linear. A plot of $1/G(\text{SSB})$ against $1/[\text{SV40}]$ is in general straight (see for example Figure 2). However, many observations have shown that a plot of $1/G(\text{SSB})$ against $[\text{DMSO}]$ exhibits a pronounced curvature (Udovicic et al. 1991; van Rijn et al. 1995; Milligan et al. 1996). It has been argued that this is a manifestation of non-homogeneous kinetics (Mark et al. 1989).

A convenient solution to interpreting this behavior is to make many plots of $1/G(\text{SSB})$ against $1/[\text{SV40}]$, each at a different but constant DMSO concentration. It is possible to derive from each plot an estimate for the value of k_2 . The estimates obtained for different DMSO concentrations differ from one another. By plotting these different estimates for k_2 as a function of $[\text{DMSO}]$, it is possible to characterize in a quantitative manner the dependence of the former upon the latter. A more general result is obtained if the DMSO concentration is converted in to the $\bullet\text{OH}$ scavenging capacity, $\sigma(\bullet\text{OH})$ (see above). The data plotted in Figures 3 are examples of this analysis.

Figure 3 is a plot of $1/G(\text{SSB})$ against $1/[\text{SV40}]$ at a DMSO concentration of $5 \times 10^{-3} \text{ mol dm}^{-3}$. The slope m and intercept c obtained from the straight line fitted to the data in this figure are 1.83 MJ dm^{-3} and $-2.17 \times 10^3 \text{ J } \mu\text{mol}^{-1}$ respectively. The estimation of the slope is simple. It is however necessary to make an extrapolation in order to estimate the intercept, and this introduces an unnecessary uncertainty. The negative value for c obtained from Figure 2 is an indication that this value is a poor estimate. A more reliable method for estimating c is to recognize from equation 1 that $c = 1/G_0(\text{SSB})$, *i.e.*, the reciprocal of the SSB yield observed in the absence of any added scavenger (and therefore c should not be negative). The use of this analysis has been described by other workers (Collins et al 1994). A value of $1.6 \times 10^{-3} \mu\text{mol J}^{-1}$ has been determined for $G_0(\text{SSB})$ using the SV40 minichromosome as the DNA substrate (Milligan et al. 1993). This leads to a value for c of $6.3 \times 10^2 \text{ J } \mu\text{mol}^{-1}$. Unfortunately, because of the probable presence of residual scavengers in the SV40 minichromosome preparation and the difficulty of removing them without destroying its delicate polynucleosomal structure, in this case the method cannot be relied upon.

A more satisfactory approach is to note that the SSB yield for the SV40 minichromosome is consistently lower than that determined using plasmid DNA by a factor of *ca.* 5 over a wide range (two orders of magnitude) of $\sigma(\bullet\text{OH})$ (Milligan et al. 1993). The difference becomes smaller at very high scavenging capacities, presumably because DNA damage by the direct effect of ionizing radiation makes a significant contribution under these conditions (Watanabe et al. 2004; Douki et al. 2006). Assuming that this factor of 5 applies at the very low $\sigma(\bullet\text{OH})$ values applicable to solutions containing no added scavenger, a value of $G_0(\text{SSB})$ for the SV40 minichromosome should be equal to $1/5^{\text{th}}$ that of $G_0(\text{SSB})$ for plasmid DNA. A value of $3.5 \times 10^{-2} \mu\text{mol J}^{-1}$ is a good estimate for the latter, leading to the estimate of $7 \times 10^{-3} \mu\text{mol J}^{-1}$ for $G_0(\text{SSB})$ for the SV40 minichromosome, or equivalently of $c = 1/(7 \times 10^{-3}) = 1.4 \times 10^2 \text{ J } \mu\text{mol}^{-1}$. Note that this estimate of $7 \times 10^{-3} \mu\text{mol J}^{-1}$ is significantly greater than the measured value of $1.6 \times 10^{-3} \mu\text{mol J}^{-1}$ (Milligan et al. 1993).

Examination of equation 1 shows that the slope m of figure 3 is equal to:

$$m = \frac{k_1[\text{DMSO}]c}{k_2} \quad (2)$$

Solving equation 2 for k_2 leads to a value of $(7.1 \times 10^9) \times (5 \times 10^{-3}) \times (1.4 \times 10^2) / 1.83 = 2.7 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$. This value is plotted in Figure 3 at $\sigma(\bullet\text{OH}) = 3.6 \times 10^7 \text{ s}^{-1}$ (the scavenging capacity corresponding to $5 \times 10^{-3} \text{ mol L}^{-1}$ DMSO). Note that the higher values of c that we consider unreliable and reject (see above) would lead to 4 – 5 fold larger estimates for the value of k_2 .

Further determinations of k_2 were made at other DMSO concentrations in an identical manner. These results are also plotted in Figure 3 along with previously obtained data for single stranded DNA (Udovicic et al. 1991) and for (double stranded) plasmid DNA (Milligan et al. 1996). In all cases there is clear evidence that the rate coefficient increases with increasing $\bullet\text{OH}$ scavenging capacity. The smaller slope indicates that the effect is weaker for single stranded DNA, although it is not clear if the explanation lies in the tertiary structure of the DNA substrate or its size. The authors used a commercially available preparation derived from calf thymus, with a reported molecular weight of $1 \times 10^6 \text{ g mol}^{-1}$ (Udovicic et al. 1991) although it was very probably a heterogenous population with a wide range of lengths. Comparison of the minichromosome and plasmid results suggests that the rate coefficient for the reaction of $\bullet\text{OH}$ with DNA in the SV40 minichromosomal form slightly larger (*ca.* 2 fold) than that for $\bullet\text{OH}$ with plasmid DNA. Conformational differences

may be responsible, because a reaction involving non-homogeneous kinetics is sensitive to the geometry of the target (Mark et al. 1989).

The 5-fold smaller SSB yield in the minichromosome relative to a plasmid target indicates that the presence of the histone proteins confers a significant protection to the DNA component. The close association (Luger et al. 1997) between the histone octamer and the DNA in a nucleosome might be expected merely on steric grounds to decrease the exposure of the DNA to $\cdot\text{OH}$ attack from the solvent. There is both experimental (Hayes et al. 1990) and biophysical modeling (Běgusová et al. 2000) evidence that this is the case in the nucleosome. A higher resolution system involving a specific binding site (the *lac* operator sequence) found protection at the DNA binding site against $\cdot\text{OH}$ attack by the *lac* repressor (Běgusová et al. 2001). All these reports are consistent with our observations.

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Figure 1.

Loss of supercoiled SV40 with increasing radiation dose. An aerobic solution containing the SV40 minichromosome ($30 \mu\text{g ml}^{-1}$), DMSO ($5 \times 10^{-3} \text{ mol dm}^{-3}$), was irradiated at $2.43 \times 10^{-1} \text{ Gy s}^{-1}$ to the doses indicated. The fraction of intact SV40 remaining after each dose was determined by using agarose gel electrophoresis (see Materials and Methods). The data are fitted with a least mean square straight line of the form $y = ce^{-mx}$. From the value of the slope m , under these conditions the D_0 is 171 Gy and the SSB yield is $5.16 \times 10^{-5} \mu\text{mol J}^{-1}$ or $1.72 \times 10^{-9} \text{ Gy}^{-1} \text{ Da}^{-1}$.

Figure 2.

Effect of SV40 concentration on the SSB yield. The SV40 concentration is expressed here as the molarity of nucleotide residues. This is a double reciprocal plot of the data from Figure 2 according to competition kinetics (see Discussion). The data are fitted with a least mean square straight line of the form $y = mx + c$. Values for the slope m and the intercept c are respectively 1.83 MJ dm^{-3} and $-2.17 \times 10^3 \text{ J } \mu\text{mol}^{-1}$.

Figure 3.

Effect of $\bullet\text{OH}$ scavenging capacity ($\sigma(\bullet\text{OH})$) on the rate coefficient for the reaction of $\bullet\text{OH}$ with DNA in the form of the SV40 minichromosome (closed symbols). The data are fitted with a least mean square straight line of the form $y = cx^m$ (solid line). Also plotted in this figure are straight lines of the same type fitted to previously published data for plasmid DNA (long dash) and single stranded DNA (short dash). The values of the slopes m are 0.38 (solid line), 0.35 (long dash), and 0.20 (short dash).