Identification of a Major Pathway of Strand Break Formation in Poly U Induced by OH Radicals in Presence of Oxygen

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A reaction mechanism is presented for strand break formation in poly U induced by OH radicals in N_2O/O_2-saturated aqueous solution based on experimental results obtained with different methods.

Radiation damage of DNA in cells leads to reproductive cell death, cell deactivation, induction of mutation and to cancer. The search for the kind of damage which may be responsible for these effects has led to the hypothesis that strand break formation is one of the more serious kinds of damage [1–3]. Indeed a single strand break induced by irradiation in biologically active single-stranded DNA was found to be a lethal event [4]. Another kind of lethal damage is loss of a base [5].

High energy irradiation generates mainly OH radicals in aqueous solution especially in presence of N_2O. These radicals are responsible for the DNA damage in aqueous N_2O-saturated solution. Lethal damage caused by OH radicals in single stranded ΦX 174 DNA in oxygenated solutions consists of 39% immediate strand breaks and 61% of other lesions [5, 6]. In absence of oxygen a mechanism describing the chemical steps leading to strand break formation has been published [7, 8]. For strand break formation in presence of oxygen, with one exception accounting for minor pathways [9] no detailed chemical mechanism has been advanced as yet. In the present paper a major pathway leading to strand break formation is proposed for poly U as substrate in presence of oxygen. Poly U was chosen as simple model compound instead of DNA, because it is single stranded at room temperature, it has no base stacking and it carries only one kind of base (uracil) [10]. Due to the presence of an OH group at position 2′ of the sugar moiety it is a better model for RNA, nevertheless a good portion of the results are relevant also for DNA.

The mechanism is based on the following experimental observations obtained with poly U in aqueous, N_2O/O_2 (4:1 v/v) saturated solution at pH 6 at room temperature and a concentration of poly U $= 2 \times 10^{-4}$ M per nucleotide: G(ssb) $= 3.5 \pm 0.5$ (G is defined as the number of molecules formed or consumed per 100 eV absorbed radiation energy, ssb = single-strand break) [11]. An increase of temperature to 70 °C after irradiation does not change G(ssb) [11]. $G(O_2^-) < 1.0 \pm 0.3$ [12], G(oxygen uptake) $\cong 14$ at 5 °C [13]. Furthermore the measured rise time of the electrical conductivity of pulse irradiated poly U solutions [12] and the rate of the decay of peroxyl radicals as measured by ESR is taken into account [14]. The rate of the increase of conductivity due to the release of counterions is a measure of the rate of chain break formation [15]. At pH 6 the counterions released are mainly K^+ [15]. The rate of chain break formation in poly U has a fast (time scale $\approx$ 1 ms) and a slow (time scale 2 s) component. A similar result has been obtained measuring the change of light scattering after pulse irradiation [16]. Under our conditions the slow component is responsible for more than 80%, of the total strand break formation [12]. In the present paper only the slow component will be discussed. The slow component can be described as if it consists of two first order reactions with a small contribution of second order processes. The slowest reaction is of first order at 20 °C with $k \approx 0.25\ s^{-1}$. This value does not change with dose rate [12]. Furthermore the rate does not change with pH in the range of pH 4 to 8. The decay of peroxyl radicals observed by ESR is of first order and has a half life of ~5S at 5 °C and ≈1.4S at 20 °C [14]. For the G value for proton formation see the following paragraph.

Experimental and Results

Poly U as potassium salt was obtained from Boehringer, Mannheim, and treated as described earlier [15].

The pH was measured with a glass electrode from Radiometer, Copenhagen. ^{60}Co-γ-irradiation of 2×10^{-4} M poly U in N_2O/O_2 (4:1, v:v) saturated aqueous solution at a dose rate of 0.28 J kg^{-1} s^{-1} at room temperature leads to a linear increase of

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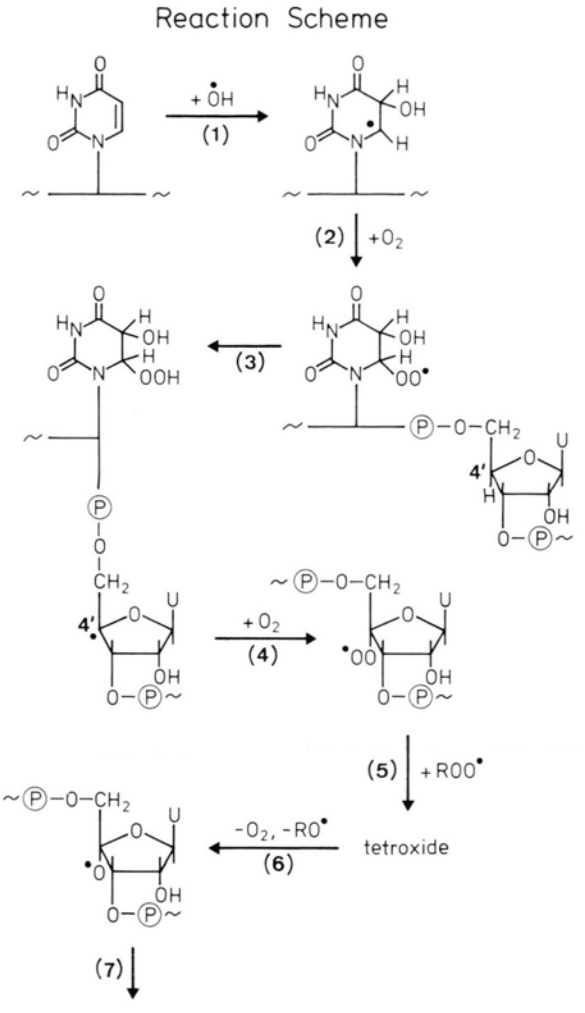
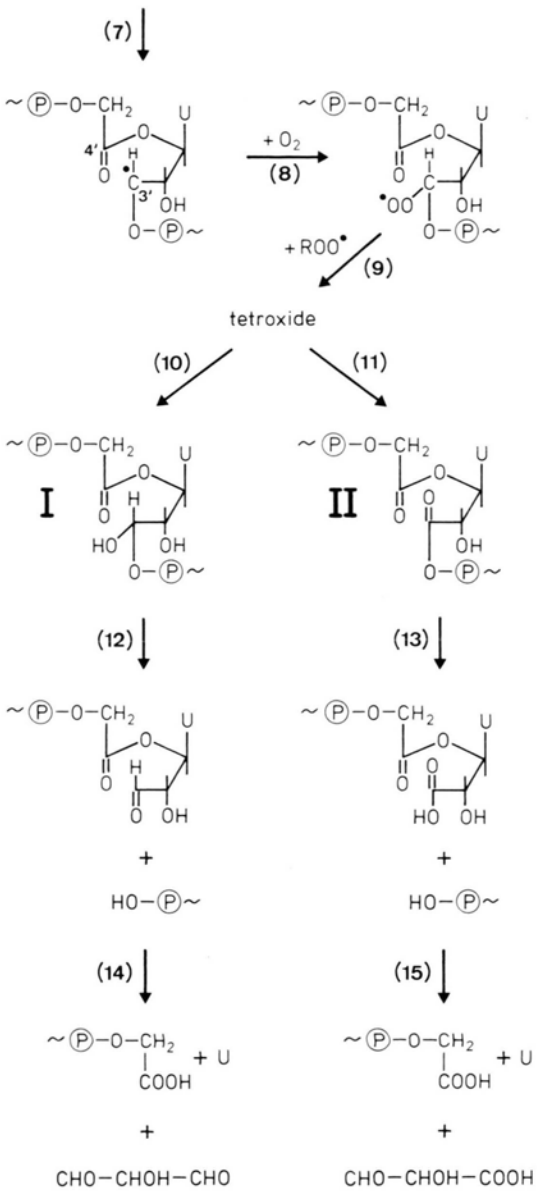
proton concentration with dose (range $0-50\ J\,kg^{-1}$). From this a $G(H^+)$ value of 2.0 ± 0.3 is obtained at pH 5.0 and 5.5. The yield of H^+ increased on standing or by heating the solution to 70 °C from 2.0 ± 0.3 to 6.0 ± 0.5. From measurements at smaller pH values with pulse radiolysis we conclude that the acid formed by irradiation has a pK value lower than 4.5. Titration of the irradiated solutions at room temperature gave $G(\text{acid}) = 5.0 \pm 0.4$.

Discussion and outline of the major pathway for strand break formation

Under the experimental conditions given only OH radicals react with the substrate. The first step is therefore the reaction of the OH radical with poly U. From studies with nucleic acids it may be assumed that more than 80% of OH add to the base and less than 20% abstract an H atom from the sugar moiety [17]. Studies with uracil in aqueous solution have shown that 80% of OH radicals add to position 5 and 20% to position 6 [18]. It is proposed that addition to the C=C bond is also the main reaction of OH radicals with the uracil moiety in poly U (reaction (1)). Addition of oxygen to these hydroxyuracil moiety radicals generates peroxyl radicals (uracil peroxyl radicals; reaction (2), only one isomer is shown). It is proposed that the peroxyl radicals abstract H atoms from the sugar moiety of a neighbouring nucleotide unit (reaction (3)). The observed rate for this reaction is in agreement with the reactivity for H abstraction reactions by peroxyl radicals as determined by Howard and Ingold [19]. For this comparison it has to be taken into account that the concentration of sugar moieties is high in the vicinity of the uracil peroxyl radical of the polymer.

Since ribose has five different kinds of C–H bonds, five different possibilities exist for H abstraction by the uracil peroxyl radical. H abstraction at position 1′ of the sugar moiety is not expected to lead to strand breaks in the course of further reactions. H abstraction at position 2′ should lead either to a fast elimination of $O_2^{\bar{}}$ after O_2 addition [20] which is not observed with a large yield, or to strand break formation by ionic elimination of H^+ and 3′-OPO_3^{--} ~ with the same rate as measured for the decay of the peroxyl radicals by ESR. Experimentally the main part of the strand break formation occurs with an average rate only slightly slower. Therefore reaction at C-2′ may contribute to a small extent. Abstraction at position 3′ and 5′ is considered to be less frequent due to deactivation by the phosphate group. This leaves position 4′ as the main reaction site. Oxygen addition to the free radical at this position, reaction (4), generates a 4′-peroxyl radical which is a tertiary one. Such radicals decay via a tetroxide (reaction (5)) which decomposes into two oxyl radicals and one oxygen molecule (reaction (6)). The 4′-oxyl radical cleaves preferentially at the C-3′–C-4′-bond (reaction (7)) since oxyl radicals react selectively [21]. Cleavage reaction (7) produces an ester of glycolic acid and a carbon radical at position 3′. The 3′ radicals add oxygen (reaction (8)) and the resulting secondary





peroxyl radicals decay bimolecularly via the Russell mechanism [22] (reactions (10) and (11)). If two 3′-peroxyl radicals are involved as shown in reactions (10) and (11) a carbonyl and a hydroxyl group will appear at the two 3′ positions in equal yields (structures **I** and **II**). The two structures are postulated to hydrolyse according to reactions (12) and (13). The rate observed for the first order contribution to strand break formation is considered to be determined by these hydrolytic reactions and/or the decay of the peroxyl radicals. The rate for hydrolysis of small molecular weight model systems, *e.g.* of the mixed anhydride of formic and phosphoric acid is found to be of the same order of magnitude [23].

In the hydrolysis (reactions (12) and (13)) two different end groups at 3′ are being formed, one with a carboxyl group and one with an aldehyde group. Therefore protons from the carboxyl group should appear during the process of strand break formation. This is observed. The protons measured at pH 5 at room temperature ($G = 2.0 \pm 0.3$) can arise only to a small extent from a newly formed phosphate end group (reaction (12) and (13)) because their pK_a value ($pK_a \cong 6-7$) is too high. Assignment of the produced protons to a stronger acid is therefore required. The proton producing acid has a pK_a value of < 4.5. This points to a carboxyl group as the origin of the measured protons since the similar glycolic acid has a pK_a value of 3.83 [24]. Since $G(H^+)$ at room temperature is approximately half of that of strand break formation ($G(\text{ssb}) = 3.5$), the main contribution to H^+ formation stems from the carboxyl group at position 3′ formed in reaction (13) and not from the phosphoglycolic acid formed in reactions (14) and (15). Otherwise $G(H^+) > G(\text{ssb})$ should have been observed. Upon heat treatment (70 °C) $G(H^+)$ rises to a value of 6.0. The additional protons are considered to arise from the phosphoglycolic acid end groups generated in reactions (14) and (15). In agreement with this it is found that the release of a substantial part of uracil also requires heating to 70 °C [25]. Further in agreement with the postulated mechanisms is the finding that protons at room temperature are produced with the same rate as strand break formation [12]. The protons measured by titration at room temperature ($G(\text{acid}) = 5.0 \pm 0.5$) represent the sum of newly formed phosphoric acid end groups ($G = 3.5$) and the $G(H^+)$ measured at pH 5 ($G = 2 \pm 0.3$). No acid is expected from the peroxyl radicals of the uracil moiety (although uracil alone gives $G(\text{dialuric acid}) = 0.9$ [26]), because the peroxyl radicals of the uracil moiety decay by H abstraction and not bimolecularly.

From the mechanism an oxygen consumption of $G(\text{oxygen uptake}) = 12$ is calculated. The values found experimentally at 5 °C increase from 14.6 up to 17.6 with decreasing dose rate and increase to 21 at 25 °C [13]. The lowest value is measured under

conditions where the contribution of chain reactions are minimized. These chain reactions do not lead to strand break formation.

Many questions which arise in the context of this mechanism will be dealt with in forthcoming publications. Only the following two questions will be treated briefly. First, why is the yield of strand break formation not around 5.3 as expected from the yield of OH radicals and the assumption that all peroxyl radicals abstract H atoms from a sugar moiety? The answer may be found in H abstraction reactions by the uracil peroxyl radicals from positions other than 4′, *e.g.* at position 1′, at the sugar which do not initiate strand breaks. In addition the 4′-peroxyl radical will react not only with another 4′-peroxyl radical. A mixed decay of peroxyl radicals might occur. This will reduce strand break formations.

On the basis of $G(\mathrm{OH}) = 5.3$ about 66% of the OH radicals lead to strand break formation and 34% do not. This shows that base radicals contribute to strand break formation, since $< 20\%$ of the OH radicals react with the sugar [17]. The slow component contributes 80% to the total strand break formation. This is the maximum possible contribution of the postulated mechanism to strand break formation.

The postulated mechanism proceeds by H abstraction at position 4′ of the sugar by the base peroxyl radical. H abstraction at positions 3′ or 5′ is expected to lead to mechanisms which proceed in principle in an analogous fashion with similar rate constants also leading to strand break formation. As already mentioned the yield of these other routes is expected to be smaller than that of the 4′ route. Some of the alternative routes have been shown by product studies to have very small yields in DNA [9]. The degree of participation of the alternative routes will be discussed in forthcoming papers.

Secondly, the yield of released unaltered free base of $G(\text{uracil}) = 3.5 \pm 0.3$ [25] has a similar value as that for strand break formation ($G(\text{ssb}) = 3.5 \pm 0.5$). Since the uracil peroxyl moiety will not be released as unaltered base, H abstraction by the uracil peroxyl radical occurs to a major extent at a sugar moiety of another nucleotide than that carrying the uracil peroxyl radical.

The release of uracil from poly U is an indication of the hydrolysis (reactions (14) and (15)) of the glycolic acid esters formed in reactions (12) and (13) because this hydrolysis renders the uracil moiety unstable towards cleavage of the N-glycosyl linkage. The hydrolysis of this ester and the release of free base (reaction (14) and (15)) are somewhat slow processes, since the formation of free base (uracil) from poly U gives the final value only after heating the irradiated poly U solutions up to 70 °C or after prolonged standing [25]. A slow release of bases from irradiated DNA was observed by Ward and Kuo [27].

Concerning the question as to whether the postulated mechanism may occur in DNA an interesting result obtained by Henner *et al.* should be mentioned [28, 29]. These authors found with DNA two kinds of 3′ end groups, a phosphate end group and with higher yields a glycolic acid end group which is attached with the hydroxy group to the phosphate at position 3′. Such a phosphoglycolic acid is the end group which is expected from the mechanism postulated in the present paper as the result of the final hydrolytic steps (14) and (15). The 5′ end group has been found by Hagen and his group [7] to account for $> 90\%$ of phosphate end groups in agreements with the now postulated mechanism.

In reaction (14) and (15) the formation of two fragments with three carbon atoms is shown. DNA in contrast to poly U does not have an OH group at position 2′. Therefore in DNA malondialdehyde would be expected as one of the products. The formation of malondialdehyde in the radiolysis of DNA in oxygenated aqueous solutions has been observed by several groups [30–32]. In case of a deoxy-nucleoside Langfinger and von Sonntag [33] have shown that the yield of glycolic acid and malondialdehyde are equal as expected from the proposed mechanism.

The above discussion shows that at least to a certain extent the postulated mechanism occurs also in DNA. However, this mechanism is not the only one in DNA since the structure of altered sugars isolated from irradiated DNA indicates that positions other than 4′ have also reacted and form alkali labile sites [9, 34].

A biologically interesting aspect of the postulated mechanism is that strand break formation and base release are connected processes. Since rejoining of strand breaks generated by enzymes *e.g.* ligases is possible without loss of biological information, not strand break formation as such but loss of a base appears to be the serious damage.

In summary, the main features of the postulated mechanisms are as follows. Peroxyl radicals of the base react by H atom abstraction with the sugar moiety from another nucleotide mainly at position 4′. Addition of oxygen at 4′ leads to a peroxyl radical which decomposes bimolecularly forming an oxyl radical which preferentially cleaves at the C-3′–C-4′ bond. The newly formed 3′ radical adds oxygen and decomposes bimolecularly mainly *via* the Russell mechanism to two structures which both hydrolyse. These hydrolytic steps constitute the formation of strand breaks. Experimental results obtained with different methods support this mechanism.

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