Hydroxyl Radical Induced Cross-Linking of Cytosine and Tyrosine in Nucleohistone

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ABSTRACT: Hydroxyl radical induced formation of a DNA–protein cross-link involving cytosine and tyrosine in nucleohistone in buffered aqueous solution is reported. The technique of gas chromatography–mass spectrometry was used for this investigation. A γ-irradiated aqueous mixture of cytosine and tyrosine was first investigated in order to obtain gas chromatographic–mass spectrometric properties of possible cytosine–tyrosine cross-links. One cross-link was observed, and its structure was identified as the product from the formation of a covalent bond between carbon 6 of cytosine and carbon 3 of tyrosine. With the use of gas chromatography–mass spectrometry with selected-ion monitoring, this cytosine–tyrosine cross-link was identified in acidic hydrolysates of calf thymus nucleohistone γ-irradiated in N_2O-saturated aqueous solution. The yield of this DNA–protein cross-link in nucleohistone was found to be a linear function of the radiation dose in the range of 100–500 Gy ($J \cdot kg^{-1}$). This yield amounted to 0.05 $nmol \cdot J^{-1}$. Mechanisms underlying the formation of the cytosine–tyrosine cross-link in nucleohistone were proposed to involve radical–radical and/or radical addition reactions of hydroxyl adduct radicals of cytosine and tyrosine moieties, forming a covalent bond between carbon 6 of cytosine and carbon 3 of tyrosine. When oxygen was present in irradiated solutions, no cytosine–tyrosine cross-links were observed.

Hydroxyl radicals (OH radicals)[1] are known to produce a variety of sugar and base lesions in DNA [for a review, see von Sonntag (1987)]. In addition, OH radicals appear to be responsible for formation of ionizing radiation induced DNA–protein cross-links in isolated chromatin and in intact cells (Mee & Adelstein, 1981; Olinski et al., 1981; Oleinick et al., 1987). The involvement of both core histone and non-histone proteins in ionizing radiation induced DNA–protein cross-linking in chromatin has been demonstrated (Mee & Adelstein, 1981; Olinski et al., 1981). DNA–protein cross-links were also formed in isolated chromatin upon treatment with H_2O_2/Fe^{2+}-EDTA, and OH radicals generated in Fenton-type reactions have been implicated for their formation (Lesko et al., 1982). Evidence indicates that the chemical bonds involved in OH radical induced DNA–protein cross-links are of a covalent nature (Mee & Adelstein, 1981; Cress & Bowden, 1983; Oleinick et al., 1987). Knowledge of the chemical nature of DNA–protein cross-links is necessary for an understanding of their mechanisms of formation and for an assessment of their biological consequences.

Recently, we described the chemical nature of a number of DNA–protein cross-links involving thymine (Thy) and various amino acids in calf thymus nucleohistone exposed to ionizing radiation in N_2O-saturated aqueous solution (Gajewski et al., 1988; Dizdaroglu et al., 1989; Dizdaroglu & Gajewski, 1989). Here, we present the evidence for OH radical induced formation of a DNA–protein cross-link involving cytosine (Cyt) and Tyr in calf thymus nucleohistone.

Experimental Procedures

Materials.[2] Cytosine, tyrosine, calf thymus nucleohistone, and phenylalanylphenylalanine (Phe-Phe) were purchased from Sigma Chemical Co. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane, acetonitrile, and 6 M HCl were from Pierce Chemical Co. Dialysis membranes with a molecular weight cutoff of 3500 were purchased from Fisher Scientific Co.

Irradiations. An aqueous mixture of cytosine and tyrosine (1 and 0.5 mM, respectively) was saturated with N_2O for 30 min and irradiated in a ^{60}Co γ-source (Gammacell-220, Atomic Energy of Canada Ltd.) at a dose of 500 Gy ($J \cdot kg^{-1}$). The dose rate of the source was 150 Gy/min, as was determined by Fricke dosimetry (Fricke & Hart, 1966). After irradiation, the sample was lyophilized. Solutions of calf thymus nucleohistone (0.35 mg/mL) in 10 mM phosphate buffer (pH 7.0) were saturated with N_2O and irradiated at doses ranging from 100 to 500 Gy. After irradiation, nucleohistone solutions were dialyzed extensively against water at 4 °C and then lyophilized.

Treatment with Hydrochloric Acid and Trimethylsilylation. Aliquots (2 mg) of lyophilized samples were treated with 1 mL of 6 M HCl in evacuated and sealed tubes for 6 h at 120 °C. Samples were lyophilized and trimethylsilylated in poly(tetrafluoroethylene)-capped hypovials (Pierce) with 0.15 mL of a BSTFA/acetonitrile (2:1 v/v) mixture by heating for 30 min at 130 °C. After cooling, samples were injected directly onto the injection port of the gas chromatography without further treatment.

Gas Chromatography–Mass Spectrometry (GC–MS). Analysis of derivatized samples were performed with a mass-selective detector interfaced to a gas chromatograph (both from Hewlett-Packard), which was equipped with an automatic sampler. The split mode was used for injections.

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[1] Abbreviations: OH radical, hydroxyl radical; Thy, Thymine; Cyt, cytosine; GC–MS, gas chromatography–mass spectrometry; Phe-Phe, phenylalanylphenylalanine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; Gy, gray ($J \cdot kg^{-1}$); SIM, selected-ion monitoring.

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endoresement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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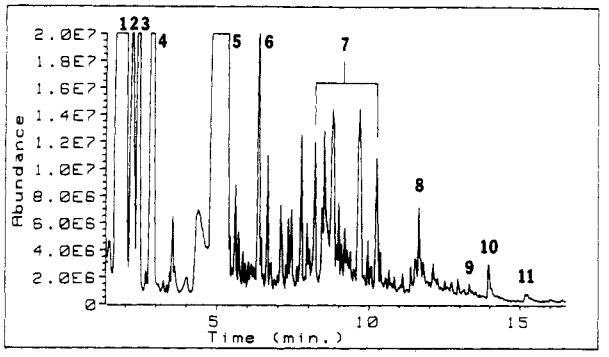


FIGURE 1: Total-ion chromatogram obtained from a γ-irradiated mixture of cytosine and tyrosine after treatment with 6 M HCl followed by trimethylsilylation. The column temperature was programmed from 150 to 270 °C at 10 °C/min after 1 min at 150 °C. For other experimental details, see Experimental Procedures. Peak identification is given in the text.

The injection port, the ion source, and the interface were maintained at 250 °C. Separations were carried out on a fused-silica capillary column (12.5 m × 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness 0.11 μm) (Hewlett-Packard). Helium was used as the carrier gas at an inlet pressure of 40 kPa. Electron-impact mass spectra were obtained at 70 eV. Selected-ion monitoring was carried out with the electron-impact mode of ionization.

RESULTS

Recently, we reported on the formation of OH radical induced DNA–protein cross-links between thymine and various aliphatic and aromatic amino acid moieties in nucleohistone in vitro (Gajewski et al., 1988; Dizdaroglu et al., 1989; Dizdaroglu & Gajewski, 1989). Calf thymus nucleohistone, a DNA–histone complex, was used as a model system for cellular chromatin. Analysis of this commercial preparation showed that it contained approximately equal amounts of DNA and protein, and gel electrophoresis revealed the presence of histones H2A, H2B, H3, and H4 (Dizdaroglu et al., 1989). In the present work, OH radical induced DNA–protein cross-linking involving Cyt and Tyr in nucleohistone was investigated. Hydroxyl radicals were generated by ionizing radiation in N_2O-saturated aqueous solution. The GC–MS technique with selected-ion monitoring (SIM) was used for identification and quantitation of DNA–protein cross-links. In order to use this technique for identification of compounds of interest in a complex system such as nucleohistone, it is essential to know the gas chromatographic–mass spectrometric properties of compounds of interest. For this reason, a mixture of Cyt and Tyr, which was γ-irradiated in N_2O-saturated aqueous solution, was investigated first. This mixture was treated with HCl prior to GC–MS analysis. The reason for HCl treatment was to have the same experimental conditions as those in subsequent experiments with nucleohistone, which was hydrolyzed with HCl prior to analysis by GC–MS/SIM.

Analysis of Irradiated Mixtures of Cyt and Tyr. Figure 1 illustrates a representative total-ion chromatogram obtained from a γ-irradiated mixture of Cyt and Tyr after HCl treatment and trimethylsilylation. Peaks 1, 3, and 5 represent the trimethylsilyl (Me_3Si) derivatives of Cyt (with two Me_3Si groups), Cyt (with three Me_3Si groups), and Tyr, respectively. Peaks 2 and 4 were assigned as Me_3Si derivatives of 5-hydroxyuracil and 5-hydroxycytosine, respectively. These compounds result from acid-induced dehydration and deamination of cytosine glycol, which is the actual OH radical induced product of Cyt (Dizdaroglu, 1986). Peak 6 represents the Me_3Si derivative of DOPA, which is an OH radical induced product of Tyr. The group of peaks designated by 7 was assigned as Me_3Si derivatives of various dimers of Cyt, and peaks 9–11 were assigned as Me_3Si derivatives of various dimers of Tyr on the basis of their mass spectra (Dizdaroglu, 1984; Karam et al., 1984). Mass spectra taken from other peaks in Figure 1 were analyzed for possible Cyt–Tyr cross-links. The product represented by peak 8 was found to correspond to a Cyt–Tyr cross-link on the basis of typical fragmentation patterns of Me_3Si derivatives of DNA bases, amino acids, and DNA base–amino acid cross-links (White et al., 1972; Leimer et al., 1977; Dizdaroglu, 1984; Gajewski et al., 1988; Margolis et al., 1988). The origin of peaks not numbered in Figure 1 is unknown.

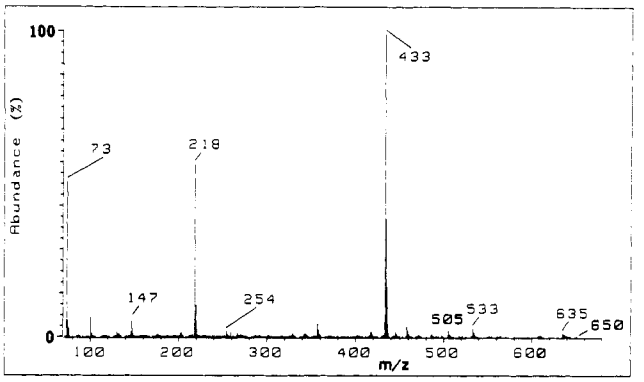
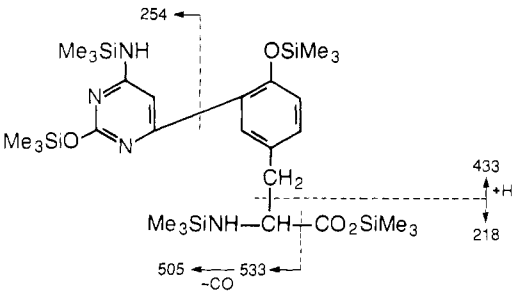


FIGURE 2: Mass spectrum obtained from the compound represented by peak 8 in Figure 1.

The mass spectrum obtained from the compound represented by peak 8 is illustrated in Figure 2. Ions at m/z 650 and 635 were assigned as the molecular ion ($M^{\bullet+}$) and the typical $(M - Me)^+$ ion, respectively. The proposed structure of this Cyt–Tyr cross-link and the fragmentation patterns leading to the characteristic ions in the mass spectrum are



Ions at m/z 73 and 147 are commonly observed with Me_3Si derivatives and are not used for diagnostic purposes (White et al., 1972). Except for the difference of 15 *mass units* in the mass of ions containing the Cyt moiety, the mass spectrum in Figure 2 is very similar to that of the Me_3Si derivative of an OH radical induced Thy–Tyr cross-link, which was reported recently (Margolis et al., 1988). In the case of the Thy–Tyr cross-link, the fragmentation patterns illustrated above have been ascertained by high-resolution mass spectrometry, and carbon 3 of Tyr has been shown to be the site of cross-linking on the Tyr moiety (Margolis et al., 1988). Because of the similarity of these two systems, it can be assumed that the site of cross-linking on the Tyr moiety is also carbon 3 of Tyr in the Cyt–Tyr cross-link as illustrated in the aforementioned structure. The site of cross-linking on the Cyt moiety is most likely to be carbon 6 of Cyt, because the unsaturated heteroatom (nitrogen 1) next to carbon 6 should facilitate cleavage of the bond between the Cyt and Tyr moieties [α-cleavage (McLafferty, 1980)], giving rise to the ion at m/z 254. Mechanistic aspects of reactions of OH radicals with Cyt,

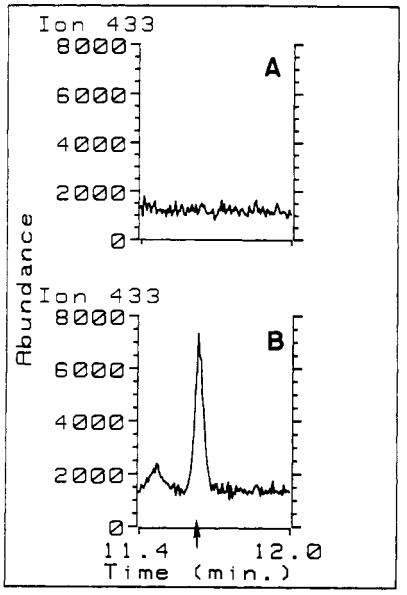


FIGURE 3: Ion-current profiles at m/z 433 obtained during GC–MS/SIM analysis of trimethylsilylated hydrolysates of nucleohistone: (A) nonirradiated; (B) γ-irradiated in N_2O-saturated aqueous solution at a dose of 200 Gy. The column conditions were as in Figure 1.

which are discussed later, strongly support this assumption. It should be emphasized that the structure of the Cyt–Tyr cross-link was inferred from the evidence discussed above, and a detailed study using nuclear magnetic resonance spectroscopy will be necessary to establish its structure unambiguously.

Identification of the Cyt–Tyr Cross-Link in Nucleohistone. Having determined the gas chromatographic and mass spectrometric properties of the Cyt–Tyr cross-link, the GC–MS technique with selected-ion monitoring (SIM) was used to search for this cross-link in trimethylsilylated hydrolysates of nucleohistone γ-irradiated in N_2O-saturated aqueous solution as previously described (Gajewski et al., 1988). A number of characteristic ions from the mass spectrum of the trimethylsilylated Cyt–Tyr cross-link (Figure 2) were monitored in the time interval where this compound is expected to elute from the GC column. Panels A and B of Figure 3 illustrate ion-current profiles at m/z 433 obtained during GC–MS/SIM analysis of trimethylsilylated hydrolysates of nonirradiated and γ-irradiated nucleohistone, respectively. The signal of the monitored ion is seen in Figure 3B at the expected retention time of the trimethylsilylated Cyt–Tyr cross-link (indicated with the arrow). Although Figure 3B illustrates only one ion, a number of characteristic ions were monitored. For identification, a partial mass spectrum was obtained on the basis of the recorded ions and their relative abundances. This partial mass spectrum is illustrated in Figure 4 and is essentially identical with the mass spectrum of the trimethylsilylated Cyt–Tyr cross-link illustrated in Figure 2, positively identifying this cross-link in nucleohistone. When mixtures of Cyt and Tyr, or nucleohistone solutions, were saturated with N_2O/O_2 prior to and during irradiation, no Cyt–Tyr cross-links were observed.

The yield of the DNA–protein cross-link involving Cyt and Tyr in nucleohistone was measured by GC–MS/SIM in the dose range from 100 to 500 Gy. For this purpose, Phe-Phe was used as an internal standard. After irradiation, dialysis, and HCl hydrolysis, an aliquot of Phe-Phe was added to an aliquot of nucleohistone samples. The samples were frozen immediately in liquid nitrogen and then lyophilized. This was followed by trimethylsilylation and GC–MS/SIM analysis during which the m/z 433 ion of the Me_3Si derivative of the

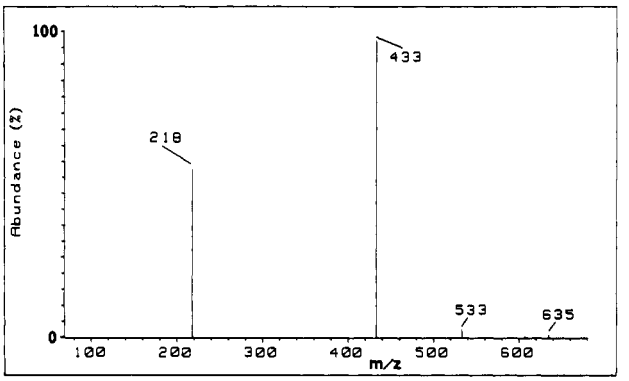


FIGURE 4: Partial mass spectrum obtained on the basis of the monitored ions and their relative intensities at the retention time indicated with an arrow in Figure 3B.

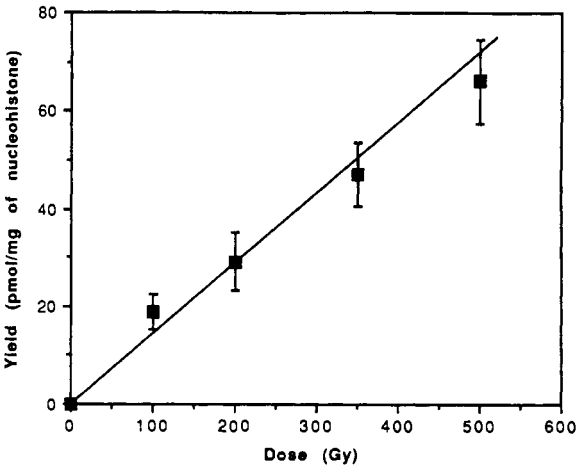


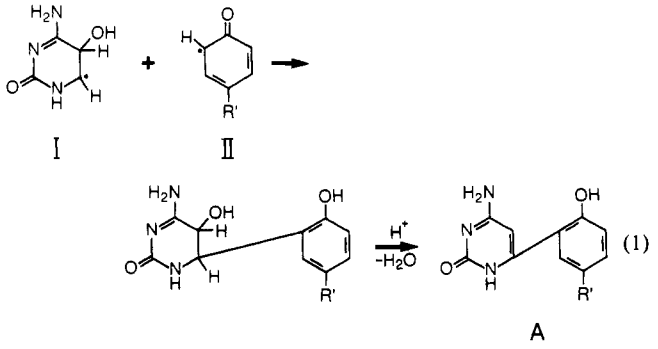
FIGURE 5: Dose–yield plot of the Cyt–Tyr cross-link in nucleohistone. Each point represents the mean (± standard deviation of the mean) from at least three independent experiments.

Cyt–Tyr cross-link was monitored along with the m/z 365 ion of the Me_3Si derivative of Phe-Phe. The quantitation of DNA–protein cross-links was described previously (Gajewski et al., 1988; Dizdaroglu et al., 1989). The yield of the Cyt–Tyr cross-link in nucleohistone was found to be a linear function of radiation dose (Figure 5). The G value (moles per 1 J of radiation energy) calculated from the linear dose–yield plot in Figure 5 was 0.05 nmol·J^{-1}. This value corresponds to formation of one Cyt–Tyr cross-link per 1 Gy in every approximately 5×10^6 nucleotides in nucleohistone. The yield of the Cyt–Tyr cross-link is the lowest among the yields, ranging from 0.06 to 8.5 nmol·J^{-1}, of the DNA–protein cross-links identified so far in nucleohistone (Gajewski et al., 1988; Dizdaroglu et al., 1989; Dizdaroglu & Gajewski, 1989).

Discussion

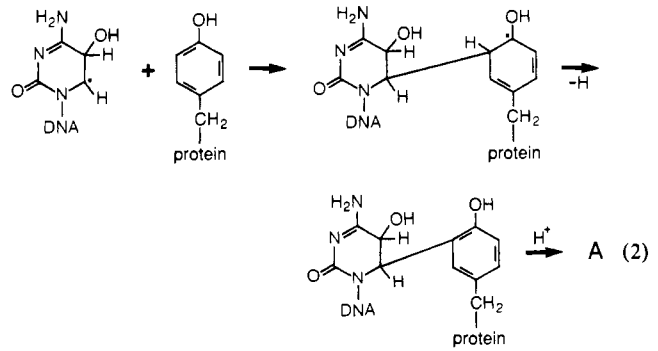
Our understanding of the mechanisms underlying the formation of the DNA–protein cross-link identified in the present work involves reactions of OH adduct radicals of Cyt and Tyr moieties in nucleohistone. Hydroxyl radicals react with Cyt by addition to the carbon 5–carbon 6 double bond with a preference for addition at carbon 5 to the extent of ≈87% (Hazra & Steenken, 1983). Hydroxyl radicals react with Tyr predominantly by addition to the aromatic ring to give dihydroxycyclohexadienyl radicals (Dorfman et al., 1962). Carbon 3 and carbon 2 of Tyr are the preferred sites of OH radical attack to the extent of ≈50% and ≈35%, respectively (Solar et al., 1984). The adduct radical formed by addition of OH radical at carbon 3 of Tyr eliminates water to give a phenoxyl radical (Land & Ebert, 1967). Combination reac-

tions of radicals formed from Cyt and Tyr are expected to account for formation of the Cyt–Tyr cross-link identified in the γ-irradiated mixture of Cyt and Tyr [R′ represents H_2C–$CH(NH_3^+)CO_2^-$]:



The dehydration step in reaction 1 is presumably catalyzed by acidic treatment. In the case of nucleohistone, a combination reaction (radical–radical reaction) of I and II, which are formed on DNA and histones, would lead to the same Cyt–Tyr cross-link A after HCl hydrolysis. Such a mechanism requires the formation of two radicals, one Cyt radical and one Tyr radical, in close proximity in nucleohistone, because of the impaired mobility of macromolecules and because of DNA–histone associations. The track model of the energy deposition of ionizing radiation in a medium provides the concept of formation of two or more radicals in track entities such as spurs and blobs (Chatterjee, 1987). Formation of such track entities containing two OH radicals in close proximity of Cyt and Tyr in nucleohistone might mediate formation of two radicals, one on DNA and one on a protein in nucleohistone, in close proximity. According to this concept, reaction 1 might represent a possible mechanism for formation of the Cyt–Tyr cross-link identified in this work. The e^- adducts of Cyt [see von Sonntag (1987)] or Tyr, which might be formed by reactions of e^- not scavenged by N_2O in spurs, might be involved in cross-linking reactions. However, such reactions would not lead to Cyt–Tyr cross-link A identified in the present work.

An alternative mechanism involving a radical addition reaction can also be envisioned for the formation of A in nucleohistone:



A radical addition reaction starting from a Tyr radical such as II and leading to A is also plausible (not illustrated here). Again, these mechanisms require formation of a Cyt radical in close proximity of a Tyr molecule, or a Tyr radical in close proximity of a Cyt molecule in the nucleohistone. Taken together, the mechanisms proposed above for formation of the Cyt–Tyr cross-link cannot be distinguished from one another by their final product. In the presence of oxygen, the formation of the Cyt–Tyr cross-link in nucleohistone was not observed. Most likely, this is due to the well-known diffusion-controlled reaction of molecular oxygen with organic radicals to give peroxyl radicals [for a review, see von Sonntag (1987)], inhibiting combination or addition reactions of Cyt and Tyr radicals.

In summary, the OH radical induced formation of a DNA–protein cross-link in nucleohistone involving Cyt and Tyr was described, and possible mechanisms underlying its formation were proposed. These mechanisms involve radical–radical or radical addition reactions of OH adduct radicals of Cyt and Tyr in nucleohistone.

Registry No. L-Tyr, 60-18-4; Cyt, 71-30-7; O_2, 7782-44-7; OH, 3352-57-6; Cyt–Tyr dimer, 124125-66-2.

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