Oxidative Damage to Cytosine: Implication for the Study of Radiation-Induced Damage to DNA

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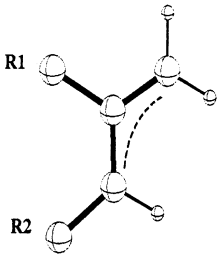
A radical observed in irradiated single crystals of cytidine, 3′-CMP, and 5′-dCMP is characterized by three anisotropic proton hyperfine couplings and is called the 3αH radical. The 3αH radical shares properties with the allyl-like radical observed in thymine derivatives. The goal of the present work is to show that the previously observed 3αH radicals are cytosine base radicals formed on 5-methylcytosine impurities in these crystals. Ab initio electron propagator calculations in the partial third-order (P3) approximation with the 6-311G(d,p) basis set have been used to show that these 5-methylcytosines are good hole traps, having an ionization potential comparable to that of guanine. The importance of these cytosine oxidation sites to the radiation chemistry of DNA is discussed.

Introduction

A radical first observed in irradiated single crystals of cytidine at room temperature by Hampton and Alexander[1] is characterized by three anisotropic proton hyperfine couplings and is

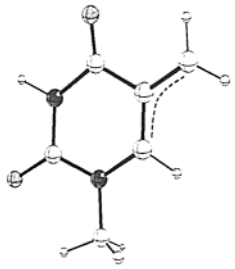


3αH Radical

therefore called the 3αH radical. This radical has also been observed in single crystals of 3′-CMP by Bernhard et al.[2] and in 5′-dCMP by Close et al.[3] Since all three molecules contain a ribose or deoxyribose, it was originally believed that this radical was formed on the furanose ring. To do so, however, the furanose moiety must change to the unsaturated form. A rather complicated scheme for the formation of the 3αH radical was described in the 3′-CMP paper,[2] but no really satisfactory reaction scheme for the formation of this radical has been put forward.

The 3αH radical shares properties with the allyl radical observed in thymine derivatives.[4] As was previously noted,[3] the hyperfine coupling tensors of the 3αH radical are very similar to those of the thymine allyl radical. In thymine derivatives, the allyl radical forms by deprotonation of the parent cation at the $>C5-CH_3$ group.[5] Of course the cytosine base does not have a methyl group, so it is not clear how the 3αH radical observed in these cytosine derivatives could actually be a base radical.

The original studies of the 3αH radical were conducted by warming the crystals after irradiation at 10 K. There was no



Thymine Allyl Radical

evidence that the 3αH radical was present at low temperatures. Newer studies have shown that the 3αH radical is present at low temperatures.[6] In cytidine, the 3αH radical is the dominant radical present in crystals irradiated and observed at 10 K. This means that any complicated schemes envisioned for the formation of the 3αH radical on the ribose or deoxyribose moiety are very unlikely to occur. It is much more likely that the 3αH radical is a primary radical that is formed in the same way as the thymine allyl radical (by irreversible deprotonation of the parent cation).

It is well known that methylation of cytosine residues within CpG dinucleotides is important in the regulation of gene expression in eukaryotes. Approximately 4% of total cytosines are methylated in vertebrate genomes.[7] Commercial supplies of cytosine nucleosides and nucleotides, which are prepared from natural products, contain C5 methylated cytosine impurities.[8] It is not commercially feasible to remove the methylated impurities. The 3αH radicals listed above were all observed in single crystals. Since crystallization is a common "purification" process, the inclusion of a small amount of methylated cytosine in these matrices was not considered in the original studies. Also, the influence of small amounts of impurities usually are undetected. However, it is known that crystals can be doped with impurities that act as selective traps of electrons or holes. The goal of the present work is to show that the previously observed 3αH radicals are cytosine base radicals formed on 5-methylcytosine impurities in these crystals and that these 5-methylcytosines may be good hole traps.

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TABLE 1: Hyperfine Coupling Tensors for the 3αH Radical in 5′-dCMP

principal values[a]	isotropic value	direction cosines			$\Delta\psi$ (deg)
proton (α1)					
−71.16		−0.8325	0.1532	0.5324	
−42.96	−44.30	0.3537	−0.5926	0.7392[b]	10.5
−18.78		−0.4264	−0.7908	−0.4392	
proton (α2)					
−67.18		0.7900	0.5955	0.1460	
−44.24	−43.52	0.3331	−0.6186	0.7132[b]	13.9
−19.15		−0.5148	0.5147	0.6856	
proton (α3)					
−34.93		0.8536	0.5065	0.1216	
−25.77	−24.13	0.2136	−0.5333	0.8051[b]	9.4
−11.69		−0.4751	0.6613	0.5805[c]	10.8

[a] All hyperfine couplings are in MHz. [b] The expected direction of A_{mid} is perpendicular to the plane, which is (0.1654, −0.5004, 0.8499). [c] The expected direction of A_{min} is in the C6−H_α direction, which is (−0.6190, 0.6353, 0.4617).

The three anisotropic hyperfine couplings for the 3αH radical observed in single crystals of 5′-dCMP are shown in Table 1.[3]

The direction of A_{min} (Table 1) is known to be associated with the direction of the >C−H bond, whereas the direction associated with A_{mid} indicates the direction of the π-electron orbital. These directions are easily calculated from the crystal structure[9] and are included in Table 1. First, it is important to determine the structure of the radical. The direction cosines listed indicate that A_{min}(α1) and A_{min}(α2) are 120° apart, whereas A_{mid}(α1) and A_{mid}(α2) are nearly coaxial. This suggests that H(α1) and H(α2) are on the same sp^2-hybridized carbon atom. Furthermore, the portion of the molecule containing the three α protons must be nearly planar since the directions of A_{min}(α2) and A_{min}(α3) are nearly identical. A planar allyl fragment incorporates all of these features.[10]

Next, the direction cosines in Table 1 can be compared with those of the known crystal structure. All three of the direction cosines associated with A_{mid} deviate approximately 10° from the computed perpendicular to the ring plane, whereas the direction of A_{min}(α3) deviates 9.4° from the computed direction of the C6−H bond. This suggests only minimal reorientation upon radical formation.[11]

The next factor to consider is the crystal structure. It is important to ask if there is room for a C5 methyl group in the crystal lattice of 5′-dCMP. The crystal structure of 5′-dCMP with a methyl group on C5 was constructed. Directions from this new carbon to all of the nearest neighbors were computed using the crystallographic program ORFEE.[12] Most of the nearest neighbors are more than 3.2 Å away. There is a cytosine C2═O only 1.81 Å away. This will likely cause a slight reorientation of the methylated cytosine. Basically, though, it seems as if there is enough room for the methyl group on C5 in the crystal lattice of 5′-dCMP. It is important to note that whereas the 5′-dCMP lattice can accommodate a few 5′-dCMP molecules with C5 methylated bases, crystals do not grow with more than about 1% of this impurity. This indicates that the methylated base impurity creates some distortions of the crystal lattice, and with too many distortions, the periodicity of the unit cell breaks down.

The same analysis was conducted on the 3αH radicals observed in 3′-CMP and cytidine. In both cases, there is room in the crystal lattice for a methylated base, and the hyperfine couplings closely fit a radical formed on the cytosine base.[13]

It appears as if the hyperfine couplings for the 3αH radical observed in these cytosine nucleosides and nucleotides correspond well with a radical formed on a 5-methylcytosine impurity in the crystals. It remains to be seen why radiation damage is trapped at the impurity site. One expects the ionizing radiation to oxidize the normal cytosine molecules in the crystal randomly. This is the case for 5′-dCMP, where the EPR signal at 10 K appears to be dominated by signals from three radical species: (1) the N3 protonated cytosine anion, (2) a cytosine cation with spin density at N1 and C5, and (3) a secondary alkoxy radical on C3′.[14] Yet in some of the crystals studied, the 3αH radical is a major defect. In cytidine, the 3αH radical is the dominant radiation-induced product at 10 K. How then does the hole defect transfer to the 5-methylcytosine impurities?

There are a number of studies where crystals of DNA bases have been artificially doped with impurities. An example would be a single crystal of cytosine monohydrate doped with 2-thiocytosine.[15] It has been shown that the oxidation product is found to be on the thiocytosine in a proportion approximately 45 times the thiocytosine/cytosine ratio in the crystal. This implies that the oxidation product is able to move about, perhaps over considerable distances, and end up in a deeper hole trap (the thiocytosine).

TABLE 2: Theoretical and Experimental IPs for the DNA Bases

base	calculated[a]	ref[b]	exptl[c]	ref
guanine (N9−H)	8.13	18	8.28	19
adenine (N9−H)	8.49	17	8.48	20
thymine (N1−H)	9.14	16	9.18	21
thymine (N1−CH_3)	8.78	16	∼8.8	21
cytosine (N1−H)	8.79	PS		
cytosine (N1−CH_3)	8.53	PS	8.65	22
5-methylcytosine (N1−H)	8.50	PS		
5-methylcytosine (N1−CH_3)	8.39	PS	8.50	22
8-oxoguanine (N9−H)	7.80	PS		
thiocytosine (N1−H)	7.46	PS		

[a] All energies are given in eV. [b] PS refers to the present study. [c] These are gas-phase vertical ionization potentials.

Theoretical Calculations

Theoretical calculations have been carried out to measure the ionization potential (IP) for normal cytosine and for 5-methylcytosine. There are lots of reports of calculated IPs for the normal DNA bases but not for 5-methylcytosine. New calculations were performed for this study.

Recently ab initio electron propagator calculations in the partial third-order (P3) approximation with the 6-311G(d,p) basis set were published for thymine,[16] adenine,[17] and guanine.[18] Excellent agreement with existing experimental data has been reported in these studies. Table 2 shows the published theoretical results and the agreement with the experimental data.[19−22] Unfortunately, there does not appear to be a P3 calculation for the ionization of cytosine, so these calculations were performed for the present study.

All calculations were performed with the Gaussian suite of programs.[23] Molecular geometries were optimized at the MP2 level using the 6-311G(d,p) basis set. No symmetry restrictions were imposed for these calculations. The results are listed in Table 2.

Table 2 shows the expected results for the normal DNA bases. All of the calculated and experimental results agree that the trend in IPs is T > C > A > G, with the pyrimidines having significantly higher ionization potentials than the purines. Guanine has the lowest ionization potential and therefore would be the easiest to oxidize. In DNA, guanine is therefore the site where most of the oxidation products are expected to reside.

For the present study, it is interesting to compare the IPs of normal cytosine and 5-methylcytosine. 5-Methylcytosine has a lower oxidation potential than cytosine. This may explain how 5-methylcytosine acts as a trap for oxidative damage in the crystals discussed above. Irradiation causes electron removal from random sites in the crystal. These holes move about and eventually encounter a 5-methylcytosine impurity in the crystal lattice. These 5-methylcytosine sites act as thermodynamic sinks in hole migration. Once the hole is trapped on the 5-methylcytosine, the cation irreversibly deprotonates at the $>C5-CH_3$ group, giving the 3αH radical, in some cases, in significant concentrations. This explains how minor impurities in the crystal can give rise to a major site of oxidative damage.

It is necessary to comment on the experimental IPs listed for cytosine and for 1,5-dimethylcytosine. The experimental paper points out that the photoelectic spectra of the cytosine derivatives are rather broad, something not seen for the other bases.[22] Also, higher probe temperatures were required for some of the cytosine derivatives (195 °C for 1,5-methylcytosine) than for the other bases (152 °C for thymine and 185 °C for adenine). This could give rise to partial decomposition. Even though no error limits were given in the experimental paper, it may be that the errors in measuring the IPs for cytosine were greater than for the other bases.

Another point to consider in reading Table 2 has to do with the protonation state of cytosine. When one looks at the experimental results for the 3αH radical in 5′-dCMP and 3′-CMP, it is important to note that the cytosine of the host material is protonated at N3. In these crystals, it is not known what the protonation state of the 5-MeC impurity is. One does know that the ionization potential of the cation of a cytosine base would be considerable higher than for the neutral base. In using Table 2 for the present discussion, it would be appropriate to consider the experimental results from neutral cytidine (where the cytosine base is not protonated at N3) and a neutral 5-MeC impurity.

One would like to have other examples as a test of the theoretical calculations. An example of doping cytosine monohydrate with thiocyosine was cited above. In Table 2, the IP for thiocytosine is given as 7.46 eV, showing that thiocytosine would be a superb hole trap in a matrix of cytosine monohydrate. The Table also contains a new calculation on 8-oxoguanine.[24] The P3 calculations have the calculated IP 0.33 eV below the calculated IP of guanine.

Relevance to DNA

The EPR spectrum of oriented DNA, irradiated at 77 K, was originally interpreted as being composed of equal amounts of thymine electron adducts and guanine electron-loss products.[25] Later studies by Sevilla[26] and by Bernhard[27] showed that a significant portion of the reduction products observed in DNA were actually composed of cytosine reduction products. Although the specific ratio of thymine/cytosine reduction products is difficult to determine, it is clear that a significant fraction of the reduction products in DNA involve cytosine, most likely N3 protonated cytosine anions.

Hüttermann and co-workers have proposed there are also thymine allyl radicals in the irradiated DNA EPR spectrum.[28] It is clear that the EPR spectra of the thymine allyl radical and the 3αH radical are nearly identical, so if it is true that the thymine allyl radical is present in irradiated DNA, then it is likely that a fraction of these radicals actually reside on the methylated cytosines.

In Table 2, one sees that the calculated and experimental ionization potentials of 1,5-dimethylcytosine are lower than that of cytosine. Actually, the ionization potential is more like that of a purine. This may have some important consequences. First, this means that some of the oxidative damage to DNA may reside on the methylated cytosines. Oxidation occurs initially at random sites. It is very likely that the electron-rich phosphates and deoxyribose moieties are initially oxidized. These holes are free to move around. In the standard model, they move until they encounter a guanine, where they are trapped. To this model one must now add the chance encounter of a hole with a methylated cytosine, where it will also be trapped. There are clearly more guanine hole traps in DNA than 5-methylcytosine traps, so one might assume that 5-methylcytosine trapping events are rare and hence insignificant. However, there is another important factor to consider.

The oxidation products discussed here are the primary radiation-induced defects. They are observed experimentally at low temperatures. One is more interested in the decay products of the primary defects that lead to the lesions observed in DNA at room temperature. This can be done experimentally by studying the free-radical reactions that the primary defects undergo upon warming the samples.

Once a hole encounters a guanine, the cation may deprotonate reversibly at N1 or at the exocyclic nitrogen on C2. The deprotonated guanine cations may simply back protonate and then recombine with an electron donor (e.g., a radical anion). There are, of course, indications that the oxidation of guanine may be important in some significant processes such as strand breaks. Although it is believed that one-electron oxidation of guanine is a likely intermediate in this process, the steps proceeding from the oxidation of guanine to strand-break cleavage are not at all clear.

However, a hole captured by a 5-methylcytosine will irreversible deprotonate at $C5-CH_3$, forming the 3αH radical. The EPR results clearly show that the 3αH radical is stable on warming. This is good evidence that the initial oxidation site is stably trapped. It remains to be seen if the 3αH radical is involved in any of the mutations linked to 5-methylcytosine, such as 5-methylcytosine deamination, which can lead to guanine−thymine mismatches. It has recently been suggested that some oxidative trapping sites (such as 8-oxoguanine and 5-methylcytosine) evolved to protect genomic DNA from more serious oxidative damage.[29]

The calculation in Table 2 has the IP of 8-oxoguanine 0.33 eV below the calculated IP of guanine, which is in line with the IP estimated by a Koopmans' theorem RHF/6-31G(d) calculation by Prat et al.[30] This suggests that 8-oxoguanine, rather than guanine, may be the ultimate hole sink in DNA.[24]

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