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Structure reactivity relationship in the reaction of DNA guanyl radicals with hydroxybenzoates

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Abstract

In DNA, guanine bases are the sites from which electrons are most easily removed. As a result of hole migration to this stable location on guanine, guanyl radicals are major intermediates in DNA damage produced by the direct effect of ionizing radiation (ionization of the DNA itself and not through the intermediacy of water radicals). We have modeled this process by employing gamma irradiation in the presence of thiocyanate ions, a method which also produces single electron oxidized guanyl radicals in plasmid DNA in aqueous solution. The stable products formed in DNA from these radicals are detected as strand breaks after incubation with the FPG protein. When a phenolic compound is present in solution during gamma irradiation, the formation of guanyl radical species is decreased by electron donation from the phenol to the guanyl radical. We have quantified the rate of this reaction for four different phenolic compounds bearing carboxylate substituents as proton acceptors. A comparison of the rates of these reactions with the redox strengths of the phenolic compounds reveals that salicylate reacts ca. 10-fold faster than its structural analogs. This observation is consistent with a reaction mechanism involving a proton coupled electron transfer, because intra-molecular transfer of a proton from the phenolic hydroxyl group to the carboxylate group is possible only in salicylate, and is favored by the strong 6-membered ring intra-molecular hydrogen bond in this compound.

Keywords

DNA damage; DNA repair; Electron transfer; Gamma-irradiation

1. Introduction

Electron removal or oxidative DNA damage is produced by the direct effect of ionizing radiation (ionization of the DNA itself) (von Sonntag, 1987; von Sonntag, 2006). Initial electron loss at other sites tends to migrate to guanine (Purkayastha et al., 2006). The site of electron loss shows a preference for guanine bases (Swarts et al., 1996; Melvin et al., 1998) presumably because this represents the most easily oxidizable site in DNA (Colson et al., 1992; Seidel et al., 1996). The resulting guanyl radical species are still strongly oxidizing in character (Steenken and Jovanovic, 1997; Shinde et al., 2009) and are subject to rapid reduction by electron donation from mild reducing agents (Willson et al., 1974; Milligan et

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al., 2001). Because this reaction restores to guanine the removed electron and prevents the guanyl radical from further chemical modification, it is sometimes described as a repair reaction. There is also evidence for adduct formation between guanyl radicals and nucleophilic amino acids (Cadet et al., 2006; Perrier et al., 2006), although it appears that this reaction is not competitive with reduction for a reagent which is capable of both (Ly et al., 2006).

In cells, naturally occurring reducing agents present in the highest concentrations would probably be glutathione (10^{-3} mol L $^{-1}$ levels) and ascorbate (10^{-4} mol L $^{-1}$ levels) (Sturgeon et al., 1998). Footprinting studies argue that the access of small diffusible species to DNA is strongly attenuated by the presence of DNA binding proteins (Pogozelski and Tullius, 1998; Begusova et al., 2000). It appears likely that electron deficient guanyl radical species located in such regions of cellular DNA closely associated with proteins would be inaccessible to antioxidants such as glutathione and ascorbate.

In these circumstances the most reactive reducing agents available for electron donation to DNA are probably those present in the residues of the amino acids comprising the binding proteins themselves. The indole, phenol, thiol, and disulfide groups present in the side chains of the amino acids tryptophan, tyrosine, cysteine, and cystine are all thermodynamically capable of reducing electron deficient guanyl radicals (Houee-Levin and Sicard-Roselli, 2001). Comparison of the observed rates of these reactions with the abundance of the amino acids argues that tyrosine residues are expected to make the largest contribution to the repair of DNA guanyl radicals not accessible by diffusible reducing agents (Milligan et al., 2004a).

If the hydroxyl group is so acidic that it is deprotonated, the resulting phenolate anion reacts very rapidly as an electron donor (Alfassi et al., 1990). But with a monomeric pK_a of 10.0, tyrosine residues are typically not extensively deprotonated under physiological conditions. There are a few proteins containing tyrosine residues which are significantly more acidic than this with pK_a s between 6 and 7 (Ly et al., 2005), but these appear to serve highly specialized purposes and are not involved in DNA binding.

However, the reactivity of the undissociated phenolic group in tyrosine as an electron donor is still affected by its acidity. The mechanism of the redox reaction appears to involve a proton coupled electron transfer (Milligan et al., 2004b; Sjodin et al., 2006; Irebo et al., 2008). The loss of the proton from the phenolic hydroxyl group occurs on the same time scale as the transfer of the electron to the guanyl radical. The reason for this mechanistic requirement is the very slow alternative reaction pathways involving energetically very unfavorable intermediates that would be formed if the electron and proton were transferred sequentially (Reece and Nocera, 2009). Indirect evidence for this coupled pathway was obtained by manipulating the strength of the phenolic reducing agent with electron donating or withdrawing substituents (Milligan et al., 2004b). We report here a more direct method using a phenolic reducing agent containing a suitably located proton acceptor that would be expected to facilitate the removal of the proton from the phenolic hydroxyl group and thus increase the rate of electron transfer from the phenol (Sjodin et al., 2006). By making use of salicylic acid we are able to include a carboxylate anion which is strongly intra-molecularly hydrogen bonded in a 6-membered ring to the phenolic hydroxyl group. Structural isomers of salicylic acid which cannot form an intra-molecular hydrogen bond serve as negative controls. This system may provide a model for DNA binding proteins in which the hydroxyl group of tyrosine is hydrogen bonded to a nearby and suitably oriented proton acceptor such as aspartate, glutamate, or histidine.

2. Experimental

2.1. Biochemical

Plasmid pUC18 was grown to a large scale in an *E. coli* host. We have described previously the means for its isolation and purification (Milligan et al., 1993). The base excision repair endonuclease formamidopyrimidine-DNA *N*-glycosylase (the FPG protein) was obtained commercially (Trevigen, Gaithersburg, MD).

2.2. Irradiation

Aqueous solutions of pUC18 were aerobically gamma-irradiated with an AECL GammaCell-1000 device containing the radioisotope cesium-137 (662 keV photon). The dose rate of 288 rad min⁻¹ (4.8×10^{-2} Gy s⁻¹) was determined spectroscopically with the Fricke system and by commercially available luminescence (Landauer, Glenwood, IL). The solutions contained sodium phosphate (2×10^{-2} mol L⁻¹, pH 7.0), sodium perchlorate (6.9×10^{-2} mol L⁻¹), sodium thiocyanate (1×10^{-3} mol L⁻¹), plasmid pUC18 (100 µg mL⁻¹), and one of four phenolic compounds (zero, or 1×10^{-7} to 1×10^{-4} mol L⁻¹). These four phenolic compounds were 2-hydroxybenzoic acid (trivial name salicylic acid, 2-OH-BA); 4-hydroxybenzoic acid (4-OH-BA); 2-hydroxyphenylacetic acid (2-OH-PA); and 4-hydroxyphenylacetic acid (4-OH-PA). Their structures are depicted in figure 1. Assuming for DNA a relative molecular mass of 650 g mol⁻¹ bp⁻¹, the plasmid pUC18 is calculated to have a relative molecular mass of $2686 \times 650 = 1.75 \times 10^6$ g mol⁻¹. This is equivalent to a concentration during irradiation of $100 \times 10^{-6} \times 10^3 / 1.75 \times 10^6 = 5.7 \times 10^{-8}$ mol L⁻¹ pUC18 targets.

Gamma radiation doses were in the range of 20 rad to 10 krad (0.2 to 100 Gy). After irradiation each aliquot (7 µL) was diluted with a solution (20 µL) containing sodium phosphate (9.8×10^{-3} mol L⁻¹, pH 7.0) and sodium perchlorate (1.6×10^{-2} mol L⁻¹) to produce final phosphate and perchlorate concentrations of 9.9×10^{-2} and 3×10^{-2} mol L⁻¹ respectively.

2.3. FPG incubation

After gamma irradiation and a subsequent dilution, the resulting solutions were incubated with the FPG protein (3 µL) at a final concentration of 3 µg mL⁻¹ (30 units mL⁻¹). They were incubated for 30 min at 37 °C. FPG is present in excess under these conditions. Additional samples were incubated under identical conditions in the absence of any added FPG protein. Because of the lower SSB yield, they usually required larger gamma irradiation doses.

2.4. Adduct formation

After gamma irradiation and subsequent dilution, the resulting solutions were incubated at 90 °C for 30 min. These conditions have been employed previously to detect guanine adducts (Ly et al., 2006).

2.5. Strand break yield

After irradiation and incubation, the plasmid was assayed for strand breaks by using agarose gel electrophoresis. We have reported previously on the means of digital video imaging of ethidium fluorescence to quantify the yield of single strand breaks (SSB) in the plasmid. Briefly, the D_0 dose of gamma rays is defined as the dose required to decrease by a factor of e the fraction of SSB-free plasmid. This dose is numerically equal to the reciprocal of the slope m of a straight line fitted to a semi-logarithmic yield dose plot. Assuming a Poisson distribution of SSBs the D_0 dose produces a mean of one SSB per plasmid, so that the

concentration of the SSB product is equal to that of the plasmid target (during irradiation $5.7 \times 10^{-8} \text{ mol L}^{-1}$, see above). The radiation chemical yield (which is called a *G*-value) is calculated by dividing this concentration by the value of D_0 .

3. Results

Figure 2 shows the loss of the supercoiled form of the plasmid pUC18 after gamma irradiation in the presence of thiocyanate ions, and a subsequent incubation with or without the FPG protein. This loss is due to the introduction into the plasmid of single strand breaks (SSB). If 4-hydroxybenzoate is present at micromolar levels during the irradiation, it has no effect on the SSB yield in the absence of FPG (not shown). The SSB yield observed under these conditions is ca. $5.2 \times 10^{-2} \text{ } \mu\text{mol J}^{-1}$ and depends only on the competition between millimolar thiocyanate and the plasmid for the hydroxyl radicals produced by water radiolysis. However, the presence during irradiation of 4-hydroxybenzoate does exert a large protective effect against the subsequent FPG induced loss of the supercoiled form. This effect is observed as a decrease in the slope of the yield dose plot shown in figure 1. Under these conditions, the supercoiled form of the plasmid is converted to its relaxed or open circle conformation by the introduction of DNA single strand breaks (SSB). From the slope of the yield dose plot, it is possible to calculate the radiation chemical yield of SSBs both with and without FPG incubation (see Experimental). The difference between these SSB yields represents the yield of DNA damage recognized by the FPG protein. This radiation chemical yield of FPG sensitive sites, $G(\text{FPG})$ was quantified for many different concentrations of 4-hydroxybenzoate in the range of 10^{-7} to $10^{-4} \text{ mol L}^{-1}$. According to competition kinetics (Spinks and Woods, 1990), the reciprocal of this yield is plotted in figure 3 against the concentration of 4-hydroxybenzoate (4-OH-BA) present during gamma irradiation.

The slope of the straight line fitted to this competition plot may be interpreted in terms of the reactivity of 4-hydroxybenzoate under the irradiation conditions (see Discussion). Three other plots (not shown) of this type were constructed using instead of 4-hydroxybenzoate one of three other structurally related phenolic compounds: 2-hydroxybenzoate (2-OH-BA, commonly called salicylate), 4-hydroxyphenylacetate (4-OH-PA), or 2-hydroxyphenylacetate (2-OH-PA).

There was no evidence for adduct formation between the plasmid and any of the phenolic compounds as judged by the absence of SSB formation after treatment at elevated temperatures (not shown).

4. Discussion

Gamma irradiation in the presence of thiocyanate ions results in single electron oxidation of the guanine bases in the plasmid (Milligan et al., 2000). The resulting radical cation deprotonates to form a DNA guanyl radical, DNA-Gua(-H) $^{\bullet}$ (Candeias and Steenken, 2000; Anderson et al., 2006). DNA guanyl radicals have lifetimes in the range of seconds (Hildenbrand and Schulte-Frohlinde, 1990; Shafirovich et al., 2001) under favorable conditions before they are trapped by reaction with water (reaction 1). The major stable product of this reaction is 8-oxoguanine (DNA-8-oxoGua) (Cadet et al., 2008), which is converted to a SSB by FPG incubation (reaction 2) (Stivers and Yang, 2003). DNA guanyl radicals are however labile in the presence of mild reducing agents which are capable of donating electrons to them (Willson et al., 1974; Milligan et al., 2001) and also reactive with nucleophiles to form adducts (Cadet et al., 2006; Perrier et al., 2006). The phenolic compounds such as 4-hydroxybenzoate (4-OH-BA) that we have used here are examples of

such mild reducing agents, but there was no evidence for adduct formation. The product of this reaction is the initial guanine base (reaction 3).

Under the irradiation conditions we have used, we argue that this is the means by which the presence of the phenolic compound during irradiation decreases the yield of SSBs observed after a later FPG incubation. The plot in figure 3 can be interpreted quantitatively using equation 1 to describe the competition between reaction 1 (trapping) and reaction 3 (repair) for the DNA guanyl radical. In equation 1, k_1 , k_3 , $G(\text{FPG})$, and $G_0(\text{FPG})$ represent the rate constants of reactions 1 and 3, and the yields of FPG sensitive sites in the presence and absence of 4-OH-BA. Therefore the value of k_3 can be calculated as $k_3 = k_1 m/c$, where m and c are the slope and intercept of the fitted straight line in figure 2. An approximate experimentally determined value of $k_1 = 0.2 \text{ s}^{-1}$ is available in the literature (Hildenbrand and Schulte-Frohlinde, 1990).

$$\frac{1}{G(\text{FPG})} = \frac{1}{G_0(\text{FPG})} \times \left\{ 1 - \frac{k_3[4 - \text{OH} - \text{BA}]}{k_1} \right\} \quad (1)$$

Using this method, the values of k_3 determined for 2-OH-BA, 4-OH-BA, 2-OH-PA, and 4-OH-PA are respectively 5.9×10^5 , 2.1×10^5 ($= 0.2 \times 1.9 \times 10^7/19$, from figure 3), 1.5×10^5 , and $1.7 \times 10^5 \text{ L mol}^{-1} \text{ s}^{-1}$.

It is not possible to interpret these k_3 values without a knowledge of the reduction potentials of the four compounds under the irradiation conditions, and also of the general behavior of the dependence of k_3 upon the reduction potential, which is well-known to be non linear (Marcus and Sutin, 1985; Graige et al., 1996). Data is available in the literature for the former (Sjodin et al., 2006), and we have reported previously on the latter by using simple phenols in this system (Milligan et al., 2004b).

Assuming that DNA guanyl radicals react with phenolic compounds we have used here by the same proton coupled electron transfer mechanism as simple phenols appear to do, it is possible to calculate the driving forces ΔG for reaction 3 for 2-OH-BA, 4-OH-BA, 2-OH-PA, and 4-OH-PA (Milligan et al., 2004b). The values of k_3 for these four compounds are plotted in figure 4 against these driving forces along with our previously reported results for simple uncharged mono-substituted phenols. The data for these simple phenols is fitted with the theoretically expected parabola (Marcus and Sutin, 1985). Three of the four carboxylated phenols we have examined in this study are significantly less reactive than uncharged phenols of similar reducing strength (similar ΔG values in figure 4). This seems reasonable since they must overcome an electrostatic repulsion in reacting with DNA, which is itself negatively charged.

Comparing the benzoic and phenylacetic acids with one another suggests that structural differences between ortho- and para-hydroxy substitution are unimportant for the phenylacetic acids, since they have very similar driving forces and react with very similar rate constants. For the benzoic acids, the 2-hydroxy derivative is a weaker reducing agent than the 4-hydroxy derivative by 11 kJ mol^{-1} , and (given the trend observed for simple phenols) would be therefore be expected to react more slowly by about 3-fold. In practice it reacts about 3-fold faster. We would argue that this difference cannot be ascribed simply to the ortho/para substitution difference, but instead to the intramolecular 6-membered ring hydrogen bond which exists in 2-OH-BA but not in 2-OH-PA (figure 1). This hydrogen bond is responsible for the phenolic hydroxyl group in 2-OH-BA exhibiting a 16,000-fold weaker acidity than in 4-OH-BA (Sjodin et al., 2006). The increase in size to a 7-membered ring destabilizes this hydrogen bond to such an extent that 2-OH-PA is only 4-fold less acidic than 4-OH-PA (Sjodin et al., 2006). The conclusion is that this hydrogen bond facilitates proton transfer away from the phenolic OH group and thereby increases the rate of the electron transfer reaction from the phenol to the DNA guanyl radical, since these two events appear to take place on similar time scales.

On the basis of these observations, it might be possible to speculate that tyrosine residues in DNA binding proteins might be more reactive towards the repair of DNA guanyl radicals if their hydroxyl groups are hydrogen bonded to suitable located nearby amino acid residues which are able to act as proton acceptors. Examples would be aspartate, glutamate, and histidine.

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Figure 1.

Structures of the four phenolic compounds used in this study. In comparison to its structural homologues, the unique feature of 2-hydroxybenzoic acid (2-OH-BA) is its ability to form a strong intramolecular 6-membered ring hydrogen bond between the phenolic OH group and the carboxylate anion (which is negatively charged under the experimental conditions).

Figure 2.

Yield dose plot for the loss of the supercoiled form of plasmid pUC18 caused by gamma-irradiation. The plasmid was irradiated under the conditions defined in the experimental section in the presence of the phenolic compound 4-hydroxybenzoic acid (4-OH-BA) at a concentration of $1 \times 10^{-7} \text{ mol L}^{-1}$ (light open square), or $1 \times 10^{-5} \text{ mol L}^{-1}$ (heavy open square or closed square). After dilution, the irradiated aliquots were incubated in the presence (light and heavy open square) or absence (closed square) of the FPG protein and assayed for their supercoiled content by gel electrophoresis. Each data set is fitted with a least mean square straight line of the type $y = ce^{-mx}$. From the slopes m of these fitted lines, we can calculate the D_0 doses and SSB yields for the three different irradiation and incubation conditions. The values are: (light open square) 0.97 Gy and $5.9 \times 10^{-2} \mu\text{mol J}^{-1}$; (heavy open square) 9.9 Gy and $5.8 \times 10^{-3} \mu\text{mol J}^{-1}$; and (closed square) 76 Gy and $7.6 \times 10^{-4} \mu\text{mol J}^{-1}$.

Figure 3.

Competition kinetics plot for 4-hydroxybenzoic acid. The reciprocal of the yield of sites recognized by the FPG protein is plotted against the concentration of 4-hydroxybenzoic acid (4-OH-BA) present during gamma-irradiation. The data set is fitted with a least mean square straight line of the form $y = mx + c$. The values of the slope m and intercept c of this line are respectively $1.9 \times 10^7 \text{ MJ L mol}^{-2}$ and $19 \text{ J } \mu\text{mol}^{-1}$.

Figure 4.

Dependence of the rate constant (k_3) for DNA repair by a phenolic compound against the driving force (DG) for the reaction. The phenolic compounds are: 2-OH-BA (closed square), 4-OH-BA (open square), 2-OH-PA (closed triangle), and 4-OH-PA (open triangle). Previously reported data for simple mono-substituted phenols are also included for comparative purposes (open circles). This data set is fitted with a least mean square parabola of the form $y = e^{(ax^2+bx+c)}$.