Long-distance radical cation reactions in DNA three-way junctions: inter-arm interaction and migration through the junction

U. Santhosh and Gary B. Schuster*

School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA

Received May 20, 2003; Revised and Accepted August 5, 2003

ABSTRACT

DNA three-way junctions (TWJ) are branched molecules having three ‘arms’. We studied long-distance radical cation migration in these assemblies by incorporating anthraquinone (AQ) groups linked by a covalent tether to one strand of one arm of the TWJ. Excitation of the AQ at 350 nm results in one-electron oxidation of the DNA, which generates a base radical cation. This leads to a relative inefficiency (compared with duplex DNA) strand cleavage at guanines following piperidine treatment of the irradiated samples. When the AQ is linked to the 5′-terminus of arm III by a flexible tether, gel electrophoretic analysis shows that strand cleavage occurs at the guanines in all three arms. We also investigated a TWJ in which the anthraquinone is specifically intercalated in arm III. In this case, a different pattern of strand cleavage is detected. We conclude that there are at least two mechanisms for long-distance radical cation migration in TWJs: (i) by inefficient charge hopping through the junction; (ii) by a through-space, cross-arm interaction when the AQ is on a flexible tether.

INTRODUCTION

The one-electron oxidation of DNA creates a radical cation (‘hole’) and results in an irreversible chemical reaction primarily at the 5′-G of G\textsubscript{n} (n = 2 or 3) sequences. It is important to understand the mechanism of this process as it is thought to cause mutations that can lead to disease or death (1). Experiments have shown that reaction often occurs at a G\textsubscript{n} sequence distant from the site of initial oxidation by migration of a radical cation 200 Å or more (2,3) through the intervening bases of duplex DNA (2,4–7). A thorough investigation of the mechanism of long-distance radical cation migration in DNA indicated that it proceeds according to a process we described as phonon-assisted polaron-like hopping (8–10). In this mechanism, the radical cation is delocalized over several base pairs in a localized structural distortion of the DNA. Thermal activation causes the delocalized radical cation to migrate from site-to-site under the influence of dynamical distortions of the DNA and motions of sodium counter ions and solvating water molecules, which modulate the ionization potential of the constituent bases (11).

Most studies of long-distance radical cation transport in DNA have been carried out using B-form oligonucleotides in solution. In addition, there have been reported investigations of charge transfer in guanine quartets (12), DNA triplexes (13,14), DNA/RNA heteroduplexes (15,16) and Z-form DNA (17). However, there have been only a few studies reported of charge transport in DNA structures that contain junctions (18–20), and these have yielded contradictory results.

DNA junctions are branched molecules that arise as intermediates in a number of DNA rearrangement processes. Four-way DNA junctions are formed during homologous recombination, whereas DNA three-way junctions (TWJs) are intermediate structures formed during triplet repeat expansion or deletion (21). These branched DNA structures are substrates for a number of enzymes, which play an important role in the later stages of recombination.

Barton and co-workers examined long-range radical cation migration in four-way junctions using both covalently and non-covalently bound rhodium metallointercalators (19). They reported that light-initiated oxidative reactions at GG steps are observed in each of the four arms of these assemblies when the rhodium metallointercalator is bound to only one arm. From these findings, they conclude that four-way junctions have relatively mobile conformations (19). More recently, Sen and co-workers (20) re-examined long-distance radical cation transport in four-way junctions that are linked to either a rhodium metallointercalator or an anthraquinone (AQ) group as the light-induced one-electron oxidant. They found that reaction at GG steps in each of the four arms occurred when the assembly contained a rhodium metallointercalator, as was observed by Barton, but reaction occurred only at GG steps in the two coaxially stacked arms when AQ was the oxidant. Sen’s investigation of this discrepancy showed that DNA duplexes and four-way junctions that contain rhodium metallointercalators aggregate in solution, which complicates the interpretation of long-distance charge transport reactions that use this oxidant.

We report here an examination of the long-distance charge transport properties of DNA TWJs using covalently linked AQ groups as the oxidant. It is known that the geometry of TWJs depends on the base sequence at the junction, the presence of...
divalent cations such as Mg$^{2+}$ (22–25), the length of the third arm (26) and extra bases that form single-stranded loops at the site of the junction (25–29). We examined perfectly base paired (i.e. no extra bases) TWJs for which atomic force microscopy (AFM) studies (30), oligonucleotide ligation-cyclization analysis (31) and fluorescence resonance energy transfer (FRET) (27) data suggest a pyramidal geometry with inter-arm angles between 60 and 90°. The arms of junctions with this structure are not normally stacked coaxially. We also examined a TWJ that incorporates a two base (T$_3$) single strand bulge, which is a more flexible assembly and thus may form structures where two arms can stack coaxially. Our results show that the site of charge injection in TWJs depends on the way the AQ is tethered to the assembly, which complicates the quantitative comparison of charge transport through junctions and simple duplex DNA.

MATERIALS AND METHODS

Materials

[γ-32P]ATP, [α-32P]ATP and terminal dinucleotide transferase (TdT) were purchased from Amersham Bioscience. T4 polynucleotide kinase (T4PNK) was purchased from New England Biolabs and stored at −20°C. Unmodified DNA oligomers and AQ-containing complementary oligomers were synthesized as described elsewhere on an Applied Biosystems DNA synthesizer (3,32). The extinction coefficients of the oligomers were calculated using an online biopolymer calculator and their concentrations in solution were determined from the absorbance at 260 nm where the AQ was replaced with adenine in the extinction coefficient determination. Reverse phase HPLC was performed on a Hitachi system with adenine in the extinction coefficient determination. CD spectra were recorded in JASCO J-720 spectropolarimeter. The gels were dried and the cleavage sites were visualized using an online biopolymer calculator and their concentrations in solution were determined from the absorbance at 260 nm where the AQ was replaced with adenine in the extinction coefficient determination. Reverse phase HPLC was performed on a Hitachi system with adenine in the extinction coefficient determination. CD spectra were recorded in JASCO J-720 spectropolarimeter.

Melting temperature ($T_m$) measurements

Melting temperatures were determined for samples consisting of equimolar amounts of DNA oligomers (2.5 μM) in buffer (1.0 ml, pH 7.0) solutions, consisting of different concentrations of sodium phosphate and magnesium acetate. Samples were placed in cuvettes (1.5 ml capacity, 1.0 cm path length) and sealed with tape to prevent evaporation of water during heating/cooling cycles. The absorbance of the samples was measured at 260 nm as a function of temperature for three consecutive runs: cooling from 90 to 10°C at a rate of 0.5°C/min, heating to 90°C followed by re-cooling at 0.5°C/min. The reported $T_m$ values are the peaks in the first derivative plots obtained by fitting the data with Microcal Origin software.

Cleavage analysis by radiolabeling and polyacrylamide gel electrophoresis (PAGE)

DNA oligonucleotides were radiolabeled according to standard procedures at either the 5'-end or 3'-end with [γ-32P]ATP and bacterial T4PNK for 5'-labeling or with [α-32P]ATP and TdT for 3'-labeling. Radiolabeled DNA was purified by 20% PAGE at 400 V. Samples for irradiation were prepared by hybridizing a mixture of unlabeled (5.0 or 8.0 μM) and radiolabeled (10 000 c.p.m.) oligonucleotides with AQ or non-AQ complementary strands (5.0 or 8.0 μM) in buffer (pH 7.0) containing different concentrations of sodium phosphate and magnesium acetate (to a total volume of 20.0 μl). Hybridization was achieved by heating the samples at 90°C for 10 min, followed by slow cooling to room temperature overnight. Samples were irradiated at ~30°C for 100 min, unless otherwise specified, in microcentrifuge tubes in a Rayonet photoreactor (Southern New England Ultraviolet Co., Barnsford, CT) equipped with eight 350 nm lamps. After irradiation, the samples were precipitated once with cold ethanol (100 μl), in the presence of glycogen (2 μl, 20 mg/ml), washed with 80% ethanol (2 × 100 μl), dried (Speedvac, low heat) and treated with piperidine (100 μl of a 1 M solution) at 90°C for 30 min. After evaporation of the piperidine (Speedvac, medium heat), lyophilization twice with water (2 μl) and dissolution in dye solution (0.25% w/v bromophenol blue, 30% v/v formamide in water), the samples (3000 c.p.m.) were electrophoresed on a 20% 19:1 acrylamide: bisacrylamide gel containing urea (7 M) at 70 W for 90 min. The gels were dried and the cleavage sites were visualized by autoradiography. Quantification of cleavage bands was performed on a Fuji phosphorimager.

RESULTS

Structures and characterization of three-way junctions

The structures of the TWJs used in this work are shown in Figure 1. TWJ(1) is composed of three DNA strands, DNA(1, 2, 3), that assemble to form a TWJ having arms I, II and III. DNA(3) contains a covalently linked AQ derivative at its 5'-end tethered with a 'short' (n = 2) or 'long' (n = 5) chain (see Fig. 2). On assembly of TWJ(1), the AQ is attached to arm III. Arms I and II of TWJ(1) are 'symmetrical' around the junction; the sequence of bases in each strand starting at the junction is the same in these two arms, however, the bases are in the opposite order, they run 5' to 3' in one arm and 3' to 5' in its counterpart. Similarly, TWJ(2) has this symmetry.

Downloaded from https://academic.oup.com/nar/article-abstract/31/19/5692/1092768 by guest on 01 January 2019

Figure 1. TWJ(1) is composed of three DNA strands, DNA(1, 2, 3), that assemble to form a TWJ having arms I, II and III. DNA(3) contains a covalently linked AQ derivative at its 5'-end tethered with a 'short' (n = 2) or 'long' (n = 5) chain (see Fig. 2). On assembly of TWJ(1), the AQ is attached to arm III. Arms I and II of TWJ(1) are 'symmetrical' around the junction; the sequence of bases in each strand starting at the junction is the same in these two arms, however, the bases are in the opposite order, they run 5' to 3' in one arm and 3' to 5' in its counterpart. Similarly, TWJ(2) has this symmetry.

Downloaded from https://academic.oup.com/nar/article-abstract/31/19/5692/1092768 by guest on 01 January 2019
Figure 1. Structures of three-way junctions.

(see Fig. 2) replaces the AQ as the one-electron oxidant, is formed from DNA(1, 5, 6). The guanines in the TWJs are identified by a subscript that indicates the number of bases from the strand 5'-terminus to that guanine. Arm III contains only isolated guanines, i.e. there are no GG steps in this arm. Arms I and II contain both isolated guanines and GG steps.

The TWJs used in this work were characterized by their non-denaturing gel behavior, Tm and by CD spectroscopy carried out in solutions containing sodium phosphate and magnesium acetate at various concentrations. The CD spectra show that each TWJ exists predominantly in a B-form DNA global structure under all conditions examined.

The Tm values of the TWJs were measured to assess the effect of structure and buffer on their stability. These data, summarized in Table 1, show that each of the TWJs studied have similar Tm values and, as expected, they are stabilized in the presence of Mg2+. Interestingly, when the AQ derivative in TWJ(1) is linked by a five carbon chain, two melting transitions are observed on the first heating cycle. A ‘normal’ melting of the TWJ at 43.8°C and a low temperature transition at 13.2°C. The low temperature transition is not seen when the AQ derivative is linked to duplex DNA with the short linker.

Photochemistry of the three-way junctions

Previous studies have shown that electronically excited AQ derivatives react with DNA either by hydrogen atom abstraction or by electron transfer, depending on whether the quinone is bound in a groove, intercalated or end capped. Hydrogen abstraction results in non-selective DNA damage (33,34), whereas electron transfer forms a radical cation that migrates through the duplex DNA and reacts with water primarily at the 5'-G of (G)n sequences (n = 2 or 3). Oxidative damage at a base is revealed as strand cleavage by gel electrophoresis and autoradiography after treatment of radiolabeled (32P) samples with piperidine. The experiments we report here were carried out at low conversion (single hit conditions) under which, on average, each DNA assembly is damaged once or not at all. Under these conditions, the amount of strand cleavage observed is proportional to the rate of radical cation migration to and from that guanine (4). A complete analysis of the migration and reaction of the radical cation in a TWJ assembly requires three independent experiments with 32P labeling on each of the three strands that compose the

![Figure 2. Structure of AQ- and UAQ-linked sensitizers.](image-url)

![Table 1. Melting temperature (Tm) data obtained from first derivative plots for three-way DNA junctions](table-url)
We performed a control experiment designed to determine if the damage observed at the isolated guanines is a consequence of the TWJ or due to the specific sequence of bases. A 5.0 μM solution of the duplex oligonucleotide formed from DNA(3) and DNA(7), radiolabeled at its 3'-end (see Fig. 6), was irradiated at 350 nm. Strand cleavage in the duplex is clearly detectable after 10 min of irradiation. Significantly, cleavage is observed at G_{14} and G_{19}, each of which is located 5' to an A (a reactivity pattern that is commonly observed) (33), andcleavage is detected at G_{32}, and G_{26}, but in contrast to TWJ(1), no significantcleavage is detected at G_{32}, even after 100 min of irradiation. This finding shows that the reaction seen at G_{26} of TWJ(1) is a property of the TWJ and not of the base sequence of arm II.

TWJ(2)_{n=2} contains a single strand TT loop at the junction site. Analysis of TWJ structures reveals that these looped junctions are more flexible than the perfect junction, and we assessed the consequence of this increased flexibility on long-distance radical cation migration. A sample of TWJ(2) was prepared that contained a radiolabel at the 3'-terminus of DNA(3). Samples were irradiated in buffer solutions that contain sodium phosphate and magnesium acetate, respectively. All samples were treated with piperidine at 90°C for 30 min after irradiation.

Figure 3. Autoradiogram of TWJ(1)_{n=2} labeled on DNA(3) with $^{32}$P (3000 c.p.m.) at the 3'-end. Samples containing 5 μM duplex at different salt concentrations (pH 7) were irradiated at 350 nm. Lane 1 is an unirradiated control in 10 mM sodium phosphate buffer solution. Lane 2 is same as lane 1 but irradiated for 100 min. Lanes 3–6 are for samples containing (lane 3) 10 mM sodium phosphate and 1 mM magnesium acetate, (lane 4) 100 mM sodium phosphate, (lane 5) 100 mM sodium phosphate and 1 mM magnesium acetate and (lane 6) 100 mM sodium phosphate and 10 mM magnesium acetate, respectively. All samples were treated with piperidine at 90°C for 30 min after irradiation.
this position increases when Mg$^{2+}$ is present. Clearly, the flexibility and stacking arrangement of TWJs affects the amount of strand cleavage observed at G$_{26}$ of DNA(3).

In TWJ(1)$_{n=2}$ or 5 and TWJ(2)$_{n=2}$, the AQ group of the AQ is linked to the 5'-oxygen atom of the terminal deoxyribose by a tether that imparts flexibility to the structure of the conjugate. The AQ group is presumed to be end capped, i.e. close to and parallel with the terminal base pair of the duplex (4), intercalated or bound in a groove. However, the data indicate that the instantaneous position of the AQ is determined by a dynamic equilibrium that is affected by the length of the tether. In certain structural variants of TWJ(1) or TWJ(2) arm I or II may be close enough to the terminus of arm III that, with an extended conformation of the tether, the AQ group could intercalate or end cap arm I or II. In this circumstance, what appears to be a long-distance reaction [cleavage at G$_{26}$ of DNA(3), for example] could actually be a local reaction of an AQ somehow associated with arm II. To assess this possibility, we prepared and examined TWJ(3), which contains a UAQ (see Fig. 2) in place of the AQ sensitizer. Previous work in this laboratory has shown that UAQ, which has an AQ group linked through a one atom tether to the 2'-oxygen atom of a ribose, is capable of sensitizing long-distance strand cleavage in duplex DNA (3). Rather than being end capped or groove bound, the AQ group of UAQ-containing duplexes is primarily intercalated at the 3'-side of the base to which it is bound. Consequently, it is less likely that the AQ of a UAQ-containing TWJ can reach across the structure and interact directly with a 'distant' arm.

TWJ(3) is identical in base sequence to TWJ(1) except that the AQ bound to the 5'-terminus of DNA(3) is removed and replaced at T$_{26}$ of DNA(2) by a UAQ bound near to the 3'-terminus of DNA(5). Irradiation and analysis of TWJ(3) having the 3'-terminus of DNA(6) radiolabeled reveals the pattern of reactivity shown schematically in Figure 8 (see Supplementary Material). Strand cleavage is detected at isolated guanines G$_4$, G$_7$ and G$_9$ and at GG steps GG$_{14}$ and GG$_{19}$, but, in contrast to TWJ(1), not at G$_{26}$. This finding shows that specific linkage of the AQ group at the terminus of arm III affects the reaction selectivity near the terminus of arm II, which indicates some inter-arm interaction leading to strand cleavage that is independent of the path through the junction.

**DISCUSSION**

Our study of long-distance radical cation migration in TWJs expands the recently reported work on four-way junctions (19,20). A key question raised in the study of four-way junctions concerns the ability of a radical cation to migrate from one arm to another that is not stacked coaxially with the first. Barton and co-workers, using a rhodium metallointercalator as oxidant, reported that this is possible (19), while Sen and co-workers, using an AQ oxidant, suggested that it is not (20). The results reported here indicate that for TWJs the answer is not simply yes or no.

Different geometries for perfect TWJs have been suggested by different investigators (23,27,36–38). These include: (i) a 3-fold symmetric 'Y' shape with inter-arm angles of 120°; (ii) a 2-fold symmetric 'Y' shape with an acute angle of 60° between two arms and 150° between the other two adjacent arms; (iii) an asymmetric 'Y' with three different inter-arm angles; (iv) a 'T'-shaped form with two inter-arm angles of 90°; (v) a trigonal pyramid with three 60° inter-arm angles; (vi) a pyramidal form with three inter-arm angles of 90°. A recent AFM study on TWJs has shown that they are not flat but
have a pyramid-like shape (30) and that the mean angle between the arms can vary from 57 to 80°. Apart from in the T-shaped structure, which appears to be unlikely, the arms of TWJs will not normally stack coaxially.

In order to put the charge migration results in TWJs in context, it is important to note that the efficiency of strand cleavage from irradiation of the AQ-linked TWJ(1) is considerably less than is typically observed for duplex DNA. This conclusion is rooted in the observation that 100 min of irradiation is required to obtain significant cleavage at GG steps of TWJ(1) compared with just 10 min for the duplex equivalent to arms III + II. We reported previously that the quantum yield of strand cleavage for duplex DNA with an AQ linked to a 5′-end having a leading AAA sequence is ~4.5% (39). Unfortunately, the structure of TWJ(1) does not lend itself to measurement of cleavage efficiency (low conversion is required and there are too many cleavage sites that react similarly to permit separation and analysis of products). Nevertheless, it is clear that the efficiency of reaction of TWJ(1) is more than 10 times less than that expected for duplex DNA. This reduction in efficiency indicates that processes that usually play a relatively minor role in duplex DNA reactions, such as strand cleavage at a G located 5′ to an A, may become more important in TWJ(1). In fact, this is just what is observed: in TWJ(1) strand cleavage at G4, G7 and G9 of DNA(3) is more
Significantly, G34 of DNA(1) and G2 of DNA(2), which are cleavage in duplex DNA is not seen at similar sites. An ‘isolated’ guanine that is not 5¢ to an A. Efficient strand cleavage in duplex DNA is not seen at similar sites. Significantly, G34 of DNA(1) and G2 of DNA(2), which are isolated guanines in ‘equivalent’ locations on arm I of TWJ(1), do not react and the GG steps in arm I are consistently less reactive than their counterparts in arm II. In addition, if charge injection into TWJ(1) occurred exclusively at the end of arm III, where the AQ is attached, then the strand cleavage pattern should not depend on whether n = 2 or 5. The observation that the length of this linker affects the reaction outcome requires that there be at least two modes of charge injection, one of which we associate with cross-arm interactions of the AQ.

Figure 9 is a schematic representation of two possible structures for TWJ(1). Of course, in reality there is a dynamic mixture of many structures that includes the two presented in Figure 9 among them, but the observations we report can be accommodated by consideration of those shown. In isomer A, the AQ group is stacked on arm III, whereas in isomer B the AQ is stacked on or intercalated in arm II, which, because of the asymmetrical pyramidal structure of the TWJ, is suggested to be closer to the AQ-labeled terminus of arm III than is arm I. The longer linker (n = 5) gives more isomer B. This proposal accommodates the asymmetrical reactivity of TWJ(1) and may account for the unusual strand cleavage at the isolated guanines at the end of this arm if the remote AQ binds nearby.

We prepared TWJ(2) and TWJ(3) to test this hypothesis. In the latter case, UAQ replaces AQ as the one-electron oxidant. It is on a shorter tether and its location, intercalated 3¢ to the base to which it is bound, should make it less able to participate in cross-arm reactions. This seems to be precisely the case because irradiation of TWJ(3) does not result in detectable strand cleavage at the isolated guanine near the end of arm II. Significantly, strand cleavage is observed at GG14 and GG19 of DNA(6), which indicates that long-distance charge migration can occur from arm III to arm II even though they are not normally stacked coaxially. However, the reaction efficiency of TWJ(3) is also much lower than is typical of duplex DNA and the reaction at G14, G7 and G6 of DNA(6) is about the same as that seen at G13G and G18G, which indicates again that the junction presents a barrier to radical cation migration.

Similar conclusions are reached from examination of TWJ(2), which differs from TWJ(1) by the presence of two unpaired thymine bases at the junction. The presence of such additional bases changes the structure of the TWJ from pyramidal to the more extended T-shaped structure. Gel electrophoresis studies have demonstrated that these bulged junctions have greater stability and can undergo ion-induced conformational change (25,40). The results of irradiation of TWJ(2) are subtly different from those of TWJ(1). The junction still presents a barrier to radical cation migration, since the reactivity is similar to TWJ(1) and G4 and G7 are at least as reactive as GG14 and GG19 of DNA(3). Strand cleavage is detected at the isolated guanine of arm II, but it is far less efficient than it is for TWJ(1) and it increases when Mg²⁺ is present in the solution. These findings show that the efficiency and path for radical cation migration depends upon certain structural details of the TWJ assembly.

CONCLUSION

Analysis of the migration of radical cations in assemblies containing TWJs is complex because two paths operate. One path appears to proceed through the junction and presumably requires at least transient coaxial stacking of the junction arms. We conclude that this path has low efficiency because much longer irradiation times are required to achieve comparable reaction than for corresponding duplex DNA. The second path bypasses the junction and occurs as a result of a through-space interaction of an AQ linked by a flexible tether to arm III with another arm of the TWJ. The evidence supporting the existence of this second path is circumstantial but convincing: the degree of cross-arm reaction depends on the length and flexibility of the tether and the way the AQ is bound to the DNA. The existence of two paths for long-distance reaction complicates the analysis of radical cation transport through the junction and no quantitative conclusions concerning the nature of radical cation transfer through DNA junctions can be reached. However, these results shed light on the previous reports concerning four-way junctions and on radical cation migration in DNA generally. It is possible that cross-arm interactions can also occur in some four-way junctions, which may explain the different results obtained with metallointercalators (19) and AQs (20) as the electron acceptor.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.
ACKNOWLEDGEMENTS

We thank Dr Sriram Kanvah of Georgia Institute of Technology for his assistance in the preparation of DNA samples and for a critical reading of this manuscript. This work was supported by the National Science Foundation and by the Vassar Woolley Foundation, for which we are grateful.

REFERENCES