Definitions and analysis of DNA Holliday junction geometry

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ABSTRACT

A number of single-crystal structures have now been solved of the four-stranded antiparallel stacked-X form of the Holliday junction. These structures demonstrate how base sequence, substituents, and drug and ion interactions affect the general conformation of this recombination intermediate. The geometry of junctions had previously been described in terms of a specific set of parameters that include: (i) the angle relating the ends of DNA duplexes arms of the junction (interduplex angle); (ii) the relative rotation of the duplexes about the helix axes of the stacked duplex arms (J_roll); and (iii) the translation of the duplexes along these helix axes (J_slide). Here, we present a consistent set of definitions and methods to accurately calculate each of these parameters based on the helical features of the stacked duplex arms in the single-crystal structures of the stacked-X junction, and demonstrate how each of these parameters contributes to an overall conformational feature of the structure. We show that the values for these parameters derived from global rather than local helical axes through the stacked bases of the duplex arms are the most representative of the stacked-X junction conformation. In addition, a very specific parameter (J_twist) is introduced which relates the relative orientation of the stacked duplex arms across the junction which, unlike the interduplex angle, is length independent. The results from this study provide a general means to relate the geometric features seen in the crystal structures to those determined in solution.

INTRODUCTION

The four-stranded DNA complex first proposed by Holliday (1) and now bearing his name has long been recognized as an important intermediate structure involved in many cellular processes, including homologous recombination (2) and repair of and replication through DNA lesions (3,4). Increasing evidence suggests that many disease- and cancer-associated proteins such as the BLM gene product and BRCA2 gene product associated with Bloom’s syndrome and breast cancer, respectively, also act to promote or suppress recombination activity (5,6), suggesting a greater role for the four-stranded Holliday junction in certain disease states. A number of single-crystal structures of Holliday junctions have been solved in the last several years, demonstrating the variability in its conformation [reviewed in Eichman et al. (7) and Ho and Eichman (8)]. Of these, the all-DNA Holliday junction structures show how the intrinsic conformation of the junction varies according to sequence, base modification and interactions with drugs and ions, and how these may perhaps affect the accessibility of the structure to proteins. We describe here methods to accurately and consistently define a set of parameters for such junctions that allow us for the first time to definitively compare the general geometry of these single-crystal all-DNA junction structures.

It has long been recognized that the geometry of Holliday junctions is highly variable, and that this variability may be important for its functional role in biology [reviewed in Lilley (9), Lilley and White (10) and Hays et al. (11)]. In solution, the two forms of the Holliday junction that have been observed are the open-X form, which is favored in low-salt conditions, and the stacked-X form, which is observed under physiological concentrations of mono- and multivalent cations (Fig. 1). The stacked-X structure is characterized by having the arms paired to form nearly continuous B-DNA duplexes, interrupted only at the junction crossovers. The arms can undergo a conformational isomerization where the stacked duplexes can switch partners dynamically (12–14), and apparently involves unstacking of the duplex arms into the open-X conformation. The transition from the stacked-X to open-X structure also provides a means for the junction to freely migrate along sequences that are inverted repeats (the antiparallel stacked-X junction proposed from solution studies is structurally locked and cannot migrate) (15,16). It is not surprising, therefore, that the structures of the junctions in complex with proteins that require scanning along a sequence are in this open-X conformation (17–20).

By combining solution studies with molecular modeling, a model of the stacked-X junction was derived in which the coaxially stacked arms are related by a positive rotation through the junction crossover (positive being defined by holding one stacked duplex in front fixed, and rotating the back duplex in a right-handed direction) (11). This interduplex angle (IDA) has been estimated directly by low-resolution methods such as gel mobility, atomic force microscopy (AFM) and fluorescence resonance energy transfer (FRET) spectroscopy (Fig. 2a) as ~60°.
More details of the DNA Holliday junction have been revealed in the past 5 years from X-ray diffraction studies on single crystals [reviewed in Eichman (7), Ho and Eichman (8) and Hays et al. (11)]. All of the single-crystal structures (16,21–24) to date are in the antiparallel stacked-X form and generally conform to the model derived from solution studies (25). One of the most surprising aspects of these structures is that they crystallize as junctions at all, since, for the most part, these are inverted repeat sequences. The sequence motif that crystallizes as junctions can be generalized as the decanucleotide d(CCNNPuCPyGG), where Pu is either an adenine or guanine purine nucleotide and Py is a cytosine, 5-methylcytosine or 5-bromouracil pyrimidine nucleotide, with the Pu6C7Py8 trinucleotide representing the core junction motif. The terminal C-G base pairs are not essential in that they can be replaced by T-A base pairs (23). The strands of the junction cross over between N6 and N7 of this core, and it is the interactions within this core set of nucleotides that are important for fixing the junction (11).

The single-crystal structures, when taken in comparison, reveal that the geometry of the stacked-X junction form is dependent on the atomic interactions around the Pu6C7Py8 core trinucleotide (11). The geometry of structures, for example those of the sequences d(CCGGTACC GG) (21,26) and d(CCGGCGCCGG) (24), in which there are direct hydrogen bonding interactions that link the major groove surface of these nucleotides to the phosphates of the junction crossover, are nearly identical, and these have served as the reference conformations with which others could be compared. When there are one or more solvent molecules bridging these hydrogen bonding interactions, the geometry becomes more variable. This prompted us to define two additional parameters, Jslide and Jroll, to describe the effects of these sequence-dependent interactions on the conformation of the junction. Jslide is defined as translation of the duplexes along their respective helix axes either toward or away from the junction crossover (Fig. 2b). Jroll (Fig. 2c) is defined as a rotation of the two stacked duplexes about their respective helix axes, leading to a change in accessibility to the major grooves of the duplexes. Together with the IDA, these parameters have been used to characterize the geometry of the Holliday junction.

Although the crystal structures are very similar in topology to the models of the stacked-X junction developed from non-crystallographic methods (25,27–32), there was one very obvious and troubling inconsistency: the crystal structures all show much shallower IDAs (~41°) in comparison with the ~60° angle previously reported. We should recognize that the solution studies provide a range of angles that are consistent with the data; however, the IDAs reported for the crystal structures fall outside these ranges. In addition, AFM measurements of sequence-immobilized junctions confirmed the broader angle (30). These apparent discrepancies have raised some questions concerning the generality of the single-crystal conformations as models for the junction in solution.

Does this difference arise as a consequence of the specific intramolecular interactions that fix an otherwise mobile junction? A recent study applying AFM to sheets of junctions that incorporate the original ACC trinucleotide core suggests that it does (33). The results showed that the ACC core sequence indeed was associated with a shallow ~43° angle relating the stacked duplex arms, and that the junction crossover was located exactly where the crystal structures indicate. Still, we are interested in relating the geometry
among crystal structures and between the crystal and non-crystal junction models and, therefore, we need a set of consistent and accurate definitions for the geometric parameters.

In earlier studies, the geometric parameters $J_{\text{slide}}$ and $J_{\text{roll}}$ were determined by superimposing each structure on that of d(CCGGTACCGG), which served as the ‘undistorted’ reference. This comparative approach is highly dependent on how the structures are superimposed, and which atoms were used to define the axes for rotations and translations. Here, we present more robust methods for the calculation of $J_{\text{slide}}$ and $J_{\text{roll}}$ that are based on the helix axes as defined by the available nucleic acid structure analysis programs CURVES (34) and 3DNA (35). The methods are entirely self-consistent, requiring only the Protein Data Bank (PDB) coordinates of an individual structure to characterize its geometry.

In addition, we re-examined the IDA and how it was determined experimentally to try to see how it is related to the conformational geometry of the crystal structures. The angle as it is estimated by FRET studies (27–29), for example, reflects a more global feature of the junction than is evident from the single angle that has been reported. In fact, the FRET measurements do not determine a single angle, but define the trigonometric relationship between the ends of each of the four arms extending from the junction. These are reduced to a single angle that is the angle between coaxially stacked duplex arms only in the specific case of the stacked-X form of the junction. However, if we retain the experimental definition of the IDA from the FRET measurements, which is the angular relationship between the ends of the arms, then the molecular center of the junction becomes the vertex of each angle. This broader definition has utility in that it becomes a more global measure of the junction geometry that is applicable not only to the stacked-X, but also to the other geometries as well. To fully exploit this feature, one must define the IDA, which is now the interduplex arm angle, for each pair of arms. In this way, we can specify not only geometry, but also direction. For example, the ideal square planar open-X form of the junction would have four identical IDAs of 90° for each angle relating two spatially adjacent arms, and two IDAs of 180° for the two pairs of diametrically opposed arms. A hypothetical tetrahedral junction would have four identical sets of IDAs at 109.5°. These are analogous to the trigonometric relationships that provided the early models of the junction in low and high salt solutions (29,36). For simplicity of discussion, we reduce the IDA back to a single unique angle in the stacked-X junctions of the DNA crystal structures, recognizing that it measures the angle from one end of an arm to another, through the molecular center of the junction, and recognizing this to be right-handed in all of the crystal structures. In this analysis, the utility of the IDA is that it provides the conceptual link to the geometry determined by other experimental methods in that it is a global feature.

We introduce here a new parameter, $J_{\text{twist}}$, which is related to the IDA, but is specifically the angle of the stacked duplex arms in the stacked-X form of the junction. Our analysis shows IDA to be a general parameter that is dependent on $J_{\text{twist}}$, $J_{\text{roll}}$ and $J_{\text{slide}}$. These relationships, therefore, allow us to relate the detailed geometry among crystal structures, and the general geometry between crystal and non-crystal models of the Holliday junction in a standardized framework.

### MATERIALS AND METHODS

The coordinates of the 12 available single-crystal structures of the Holliday junction were retrieved from the Nucleic Acid Data Bank (NDB) (37) and are listed by sequence and NDB accession number in Table 1. For those structures in which only two DNA strands form the crystalographic asymmetric unit, the complete four-stranded junction was either retrieved from the NDB if available or generated by application of the appropriate symmetry operators. All calculations were performed on the four-stranded biological units unless otherwise noted. All DNA junctions to date, with only a single exception

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**Table 1.** Single-crystal DNA Holliday junction structures

<table>
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<th>Sequence</th>
<th>NDB accession number</th>
<th>Abbreviation</th>
<th>Average helical twist</th>
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<td>37.7°</td>
</tr>
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<td>37.2°</td>
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<td>UD0030 (22)</td>
<td>AChU-2Ca</td>
<td>37.8°</td>
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</tbody>
</table>

Twelve DNA-only single-crystal Holliday junction structures are listed with their sequence (the trinucleotide cores are highlighted in bold italics), Nucleic Acid Databank (NDB) accession number and reference (if published), and abbreviation used in this paper. The abbreviation lists the core N,N,N-trinucleotide sequence, the number of strands in the crystallographic asymmetric unit, the primary cation type present in the structure and, for multiple identical entries, the order in which they were listed in the NDB (11). Sequence variations outside the core trinucleotide are specified by the lower case letter, with ‘g’ indicating a G–A mismatch in the core trinucleotide are highlighted in bold italics), Nucleic Acid Databank (NDB) accession number and reference (if available or generated by application of the appropriate symmetry operators). All calculations were performed using either CURVES (34) or 3DNA (35).
(16), have been crystallized in the monoclinic C2 space group, with the dyad or pseudo-dyad running parallel to the crystallographic y-axis. To simplify this presentation, we will consider only the set of structures in the C2 crystal group.

The coordinate system and reference points

The junction coordinate system. In order to define and calculate the geometric parameters of the Holliday junction seen in DNA crystals, we first developed a general coordinate system that is based on two general planes that relate the components of the four-stranded junction. The plane that would split the junction into two separate nearly continuous B-DNA duplexes we call the resolving plane, while the plane that cuts through the stacked duplexes between the base pairs that flank the junction crossover we call the bisecting plane (Fig. 3). The intersection of the two planes is the dyad axis (crystallographic y-axis). The projection of the two helix axes onto the resolving plane defines two vectors used to calculate the geometric parameter \( J_{\text{twist}} \), while the points of intersection of the helix axes with the bisecting plane along with the junction center defines the two vectors used to determine the parameter \( J_{\text{roll}} \).

Figure 3. Definitions of reference points, axes and planes for the stacked-X DNA junction. The junction is shown with a junction center (blue sphere) sitting between the crossing strands of the stacked-X junction. Each set of stacked duplexes has its own helix axis running through the base pairs. The junction is divided into the two sets of stacked duplexes by the resolving plane (which is the y-z crystallographic plane of the single-crystal structures in the C2 space group). A second bisecting plane (the crystallographic x-y plane) divides the junction through the stacked duplexes into the top and bottom halves. The intersection of the resolving and bisecting planes defines the dyad axis (crystallographic y-axis). The projection of the two helix axes onto the resolving plane defines two vectors used to calculate the geometric parameter \( J_{\text{twist}} \), while the points of intersection of the helix axes with the bisecting plane along with the junction center defines the two vectors used to determine the parameter \( J_{\text{roll}} \).

Defining helix axes and the intersection with the bisecting plane. The stacked-X conformation pairs the arms extended from the junction into two sets of nearly continuous B-DNA duplexes. In the current single-crystal structures, each of the stacked duplexes is formed by a 4 bp arm stacked on top of a 6 bp duplex. The various geometric parameters relate the relative orientation and translation of these two sets of stacked duplexes across the junction; therefore, in order to accurately analyze these geometric parameters, we need to accurately define the helix axes that run through the base pairs for each set of stacked arms. The helix axes, both global and local, were determined for the two pairs of stacked arms from the atomic coordinates of each structure using either CURVES (34) or 3DNA (35). As 3DNA does not calculate a true global helix axis, a global helix axis was approximated from the set of local axis unit vectors by taking the mathematical average of all unit vectors running through each set of stacked B-DNA duplex arms and their associated origins. The average unit vector is then extended in both directions from the average origin to provide an approximate global axis. All other helix axes are defined by the terminal endpoints of each duplex as calculated in the appropriate analysis program.

In order to calculate \( J_{\text{roll}} \) and \( J_{\text{slide}} \), it is necessary to determine the coordinates of the two points where the individual helix axes would cross the bisecting plane (Fig. 3). The intersection of any line defined by the points \( \mathbf{a} \) and \( \mathbf{b} \) and a plane defined by the three points \( \mathbf{u} \), \( \mathbf{v} \) and \( \mathbf{w} \) is a point with coordinates \( (i, j, k) \). This point of intersection is determined by solving a set of four simultaneous equations (Equations 1 and 2) for coordinates \( i, j, k \) and \( r \) where \( \alpha_x \), \( \alpha_y \) and \( \alpha_z \) are the x, y and z components of point \( a \) (the components of \( b \), \( u \), \( v \) and \( w \) are defined similarly).
where

\[ i = a_x + (a_y - b_y)t \]
\[ j = a_y + (a_z - b_z)t \]
\[ k = a_z + (a_z - b_z)t \]

Solving Equations 1 and 2 for \( t \),

\[ t = \begin{bmatrix} 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 0 \\ u_x & v_x & w_x & b_x - a_x \\ u_y & v_y & w_y & b_y - a_y \\ u_z & v_z & w_z & b_z - b_y \end{bmatrix} \]

The coordinates \( i \), \( j \) and \( k \) for the point where the helix axis intersects the bisecting plane is thus determined by first calculating \( t \), then applying that to solve the formulae in Equation 2.

Definitions and analysis of junction parameters

The interduplex angle (IDA). The IDA of the stacked-X junction when determined experimentally (e.g. using FRET methods) measured the angles relating the ends of the helical arms. This global angle can generally be interpreted as the angle from the end of one arm to the body center of the junction to the end of the next arm. For junctions having very long arms, this essentially is the projected angle between the stacked duplexes. For the stacked-X junctions seen in the crystal structures, however, the arms are very short and, therefore, the end to body center to end angle is not equivalent to the projected angle. To be consistent with the experimental values determined in solution, the IDA is defined as the angle from the end of one stacked duplex to the end of the opposite stacked duplex, with the vertex of the angle placed at the center of the junction crossover as described above. As was discussed in the Introduction, even with the more detailed parameters described below, this global parameter remains useful as a general tool to assess the contributions of the individual components to the overall structure of the junction.

A positive IDA is defined as having the stacked duplex in the back rotated in a right-handed direction relative to the stacked duplex in the front (Fig. 2a). Note that the direction is not conveyed, but is inferred in this single angle. By this definition, calculation of IDA requires some definition of the helix axes for each of the stacked pairs of arms. Once these axes are determined from CURVES or 3DNA, the IDA is the angle relating a point at the end of the helix axis of the 6 bp arm (a) to the crossover center (c) to the end of the helix axis of the opposite 4 bp arm (b). If global helix axes are determined, this is simply the dot product of the two vectors \( \mathbf{ac} \) and \( \mathbf{bc} \). If local base pair-centered axes are defined, then points a and b used to calculate IDA are simply those axis vectors at the termini of opposing 6 and 4 bp arms, respectively.

Since the angle is determined from the helix ends, the IDA is \( \geq 0^\circ \), even when the two duplexes are in a perfectly antiparallel alignment, because the ends can never overlap. In addition, the IDA is a length-dependent parameter since, for junctions with very short arms, the IDA is highly dependent on the width of the duplex arms. Only when the arms extend to infinite length does the width no longer become relevant. Since we are using the IDA as a global measure of geometry, it makes sense that this is length dependent. We would expect, for example, that if a FRET study could be performed on such constructs with short and staggered arm lengths, such as those that have been crystallized, there would be a measurable distance between the ends of the two adjacent arms and an apparent angle relating these ends, even when they are exactly aligned.

In order to relate the group of single-crystal structures to the geometry of the junction from solution studies, we have defined a new parameter (\( J_{\text{twist}} \)), which is length independent. In previous publications, \( J_{\text{twist}} \) and the IDA were considered one and the same, but here we need to make a true distinction for how these two parameters are defined and calculated. Normally, in constructs where the lengths of the arms are very long and nearly equal in length (such as those used in electrophoretic, FRET and AFM studies), this distinction would be trivial. However, the single-crystal structures have short and uneven length arms, and the two parameters can be very different in terms of what they measure.

Definition and analysis of \( J_{\text{twist}} \). The parameter \( J_{\text{twist}} \) is defined as the angle between the two helix axes when projected into the resolving plane of the junction coordinate system. By placing the axes onto a common plane, the width of the duplex arms is no longer relevant and thus this angle becomes length independent. The other advantage of \( J_{\text{twist}} \) is that it can be measured empirically from the experimental X-ray diffraction data without any assumptions concerning helix axes. This serves as an explicit set of independent reference parameters to assess the accuracy by which various methods describe the conformation of junctions. \( J_{\text{twist}} \) can be determined directly from the \( \mathbf{a} \cdot \mathbf{b} \) plane of the crystal unit cell. This is evident from the Patterson maps, which reflect the vectorial distances between scatterers in a unit cell and, in the case of B-type DNAs, are dominated by the distances between the parallel stacked base pairs of the double helix (Fig. 2a). In the particular case of the junction structures, the Patterson map in the \( \mathbf{a} \cdot \mathbf{b} \) plane at \( c = 0 \) is dominated by the redundant base pair stacking within the duplex arms on both sides of the junction crossover and therefore is essentially the projection of the base pair planes onto the resolving plane of the junction. The X-pattern reflects the projection of the stacked duplex arms across the junction crossover as projected into the resolving plane. The \( J_{\text{twist}} \) for any structure in this particular crystal group can be accurately determined by measuring the angle between the set of stacked base planes in the Patterson map, or directly from the unit cell dimensions along the \( \mathbf{a} \) and \( \mathbf{b} \) edges by Equation 4.

\[ J_{\text{twist}} = 2\tan^{-1}(\mathbf{b}/\mathbf{a}) \]
The Patterson map also provides a very accurate measure for the average rise between base pairs in helices where the base pairs are not highly inclined, which can be used in subsequent empirical analysis of the junction geometries. In this case, the average experimental rise is simply 1/20 the length of the diagonal in the crystallographic \( a-b \) plane. In both calculations, we assume that the duplex arms of the junctions are stacked end-to-end and in the \( a-b \) plane. These are indeed the characteristics of the junctions crystallized in the C2 space group.

The parameter \( J_{\text{twist}} \) can also be calculated analytically by projecting the helix axes (local or global axes defined from CURVES or 3DNA) of the stacked duplexes onto the resolving plane (the crystallographic \( x-y \) plane, by setting \( y = 0 \)) and taking the dot product of these helix axis vectors. Determining \( J_{\text{twist}} \) from the two global helix axes simply requires that we define two vectors \( AB \) and \( CD \) that describe the ends of these axes, dividing their dot product by the product of their vectorial normals and taking the inverse cosine (Equation 5).

\[
J_{\text{twist}} = \cos^{-1} \left( \frac{AB \cdot CD}{|AB||CD|} \right)
\]

\( J_{\text{twist}} \) calculated from local helix axes is simply the inverse cosine of the average of the dot products of each possible pair of local axis vectors between the two opposing duplexes. By definition, each local axis is a unit vector and therefore the denominator of Equation 5 in each case is 1. The primary problem in this determination of \( J_{\text{twist}} \) is that it is highly dependent on how the helix axis is defined. In order to assess which form of the helix axis (local or global and whether defined by CURVES or 3DNA), we will compare the values of \( J_{\text{twist}} \) between that determined from the Patterson maps and those calculated from the various helix axes.

Definition and calculation of \( J_{\text{slide}} \). \( J_{\text{slide}} \) is defined as translation of the stacked duplexes along their respective helix axes either toward or away from the junction crossover. Previously, calculation of \( J_{\text{slide}} \) required comparison with a reference structure, typically that of ACC-4Na, which was assumed to have little or no \( J_{\text{slide}} \). The structure of interest was superimposed onto the ACC-4Na structure on the basis of five cycles. These are indeed the characteristics of the junctions crystallized in the C2 space group. Determining \( J_{\text{slide}} \) from the two global helix axes simply requires that we define two vectors \( AB \) and \( CD \) that describe the ends of these axes, dividing their dot product by the product of their vectorial normals and taking the inverse cosine (Equation 5).

\[
J_{\text{slide}} = \cos^{-1} \left( \frac{AB \cdot CD}{|AB||CD|} \right)
\]

\( J_{\text{slide}} \) is positive when the direction of the shift is toward the 4 bp arm and negative when it is toward the 6 bp arm. The factor 3.5/9 is the theoretical ratio of the short arm relative to the full length of the 10 bp stacked duplex (the junction falls between base pairs 4 and 5, which means that there are 3.5 rises between the center of the junction and the end of the 4 bp arm).

In this study, we have developed precise definitions for a set of geometric parameters (\( J_{\text{twist}}, J_{\text{slide}} \) and \( J_{\text{roll}} \)) that help us relate the details of the Holliday junction conformation to a measure of its global conformation (the IDA). From these definitions, a set of empirical and analytical methods have been devised to calculate these parameters from the atomic coordinates of single-crystal structures. The empirical methods are those that do not rely on an explicit definition of the helix axis in determining their values. For example, the empirical \( J_{\text{twist}} \) is determined from the crystallographic unit cell, \( J_{\text{slide}} \) from discrepancy between measured and expected the lengths of the arms relative to the bisecting plane, and \( J_{\text{roll}} \) from the angle of the best fit cylinders for the two pairs of stacked duplexes, all as described in Materials and Methods.

RESULTS

In this study, we have developed precise definitions for a set of geometric parameters (\( J_{\text{twist}}, J_{\text{slide}} \) and \( J_{\text{roll}} \)) that help us relate the details of the Holliday junction conformation to a measure of its global conformation (the IDA). From these definitions, a set of empirical and analytical methods have been devised to calculate these parameters from the atomic coordinates of single-crystal structures. The empirical methods are those that do not rely on an explicit definition of the helix axis in determining their values. For example, the empirical \( J_{\text{twist}} \) is determined from the crystallographic unit cell, \( J_{\text{slide}} \) from discrepancy between measured and expected the lengths of the arms relative to the bisecting plane, and \( J_{\text{roll}} \) from the angle of the best fit cylinders for the two pairs of stacked duplexes, all as described in Materials and Methods.
Table 2. Geometric parameters of DNA Holliday junction single-crystal structures

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<th>Junction</th>
<th>gACC-2Na</th>
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The geometric parameters J_twist, J_slide, J_roll and IDA have been calculated for the 12 single-crystal DNA-only Holliday junction structures listed in Table 1 by each of five methods: empirical, global and local best-fit helix axes from CURVES (34), and local and approximate global helix axes from 3DNA (35).

Analytical methods are defined here as those that utilize helical axes that are defined by either CURVES or 3DNA (34,35), two of the most widely used methods for analyzing helical conformations of nucleic acids. The analogous analytical determination of J_twist is to calculate the product of the helical axes of the stacked duplexes. In applying CURVES and 3DNA, we have used either a local axis or a global analysis. A local analysis would use specifically the vectors defined by either program that sits in the bisecting plane to describe the geometry of the junction. For J_twist, this would be the cross-product of the two axes in the bisecting plane, while the points of intersection of these local vectors with the bisecting plane are used in calculating J_roll and J_slide, as described in Materials and Methods. These values would best reflect the conformational geometry centered locally at the junction crossover without regard to the geometry of the overall junction. The analogous global axis analysis uses either the global axes defined by CURVES or an average of the vectors of the local axes from 3DNA. In either case, this global analysis (particularly when averaging the local vectors) is valid only for integral turns of the helix. In the case of the single-crystal structures available for the stacked-X junctions, the 10 bp of stacked duplex arms make approximately one full turn of the helix (the average helical twist for all structures in the study is 37.4° with an SD = 0.24° for CURVES, and 37.7° with an SD = 0.31° for 3DNA, which is 5% of a full helical turn) and, therefore, these global analyses are applicable. Since these global values reflect the conformational behavior of the overall junction, they are expected to be most analogous to the values determined empirically.

Comparison of junction geometric parameters from empirical and analytical methods

The geometric parameters J_roll, J_twist and J_slide have been calculated for each of the 12 available DNA-only Holliday junction crystal structures in the C2 space group according to the empirical and analytical methods described (Table 2). The values determined by empirical methods for J_twist, J_slide and, to a lesser extent, J_roll will serve as reference values to compare with the analytical methods that utilize the helix axes. Values are compiled for the parameters calculated using either the global or local helix axes from CURVES (34) and 3DNA (35) and are compared with those from the empirical methods to determine which analytical method is most appropriate for junctions. In addition, the IDA of each structure is determined using either the CURVES or 3DNA global axes to elucidate the contributions of J_roll, J_twist and J_slide to this more general parameter.

The basis for this assessment was not to compare the actual values for the parameters as they are derived from the empirical and analytical methods, but to compare the rank order of the structures as defined by each method. For example, the structure with the largest J_twist should be identified as such if the method of calculation truly represents the geometry of the conformation. Thus, if we accept that the empirical values for J_roll, J_slide and, to a lesser extent, J_roll are the most representative of the details of each structure, we can compare each of the rankings of these values as calculated using the global or local helix axes as defined by CURVES or 3DNA by a Spearman rank correlation. The Spearman rank correlation coefficient is defined as:

\[
r_s = 1 - 6 \sum \left[ \frac{d^2}{N(N^2 - 1)} \right]
\]

where \(d\) is the difference in pairwise statistical ranks between two equivalent values and \(N\) is the number of pairs. A Spearman rank correlation coefficient \(r_s\) near 1 or -1 indicates strong positive or negative correlation between the values, while a value near zero indicates poor or no correlation.

As shown in Table 3, when comparing Spearman rank correlation coefficients, calculations of the geometric parameters using either the local or global helix axes as
defined by CURVES are consistently better correlated to the empirically determined parameters than those from 3DNA. For the \( J_{\text{twist}} \) values, both local and global helix axes from 3DNA are anticorrelated with the empirical values. Of the CURVES values, both the global and local helix axes correlate very well with the empirical \( J_{\text{roll}} \), while \( J_{\text{twist}} \) is marginally better with the local axes. The strongest discriminator is seen with \( J_{\text{slide}} \) where the CURVES global calculations are significantly better correlated with the empirical values than those determined from the CURVES local axes, or either the 3DNA global or local axes. This is not unexpected, as these geometric parameters are global characteristics of the Holliday junction, representing rotation, translation and orientation of entire duplexes, which are best approximated by best-fit global helix axes. We conclude, therefore, that the most representative approach to analyzing these junction parameters is to use the global helix axes from the CURVES program, and it is these values that we will consider for the remainder of this discussion.

### Components of the IDA

With accurate values for \( J_{\text{twist}}, J_{\text{slide}}, \) and \( J_{\text{roll}} \) now in hand, we can assess the contribution of each of these specific parameters to the more global measure of the junction geometry, the IDA. By multivariate least-squares linear regression techniques, we can derive correlations between the IDA and each of these parameters taken singly and in combination. Of the single parameters, IDA correlates most strongly with \( J_{\text{roll}} \) and \( J_{\text{twist}} \), each with \( R^2 \) values greater than 0.99. \( J_{\text{slide}} \) by itself is a poor predictor of IDA (\( R^2 = 0.30 \)). When \( J_{\text{roll}}, J_{\text{twist}}, \) and \( J_{\text{slide}} \) are taken in combination, the three parameters together predict IDA reasonably well (\( R^2 = 0.59 \)) but not as well as \( J_{\text{roll}} \) or \( J_{\text{twist}} \) by themselves. Interestingly, of the three possible pairs of parameters used to predict IDA, \( J_{\text{roll}} \) and \( J_{\text{slide}} \) correlate best to IDA (\( R^2 = 0.57 \)), nearly as well as all three parameters together. \( J_{\text{roll}} \) does seem to play the largest role in predicting IDA from the remaining three parameters, as the correlation between \( J_{\text{slide}}, J_{\text{twist}} \) and IDA is the worst of any combination of parameters (\( R^2 = 0.33 \)).

The contributions of the individual geometric parameters to the IDA are seen most dramatically in the two junction structures ACmC-2Ca and ACBu-2Ca. The ACmC-2Ca junction has a very large \( J_{\text{twist}} \) and, consequently, one would expect it to also show a large IDA. On the contrary, it has the shallowest IDA of all (60°). We attribute this to the very large \( J_{\text{slide}} (>2 \text{ Å}) \), which shifts the stacked duplex arms in the direction of the shorter 4 bp arm. This has the effect of equalizing the positions of the ends of the 4 and 6 bp arms relative to the junction crossover and, since the IDA is defined as the junction end to center to end angle, a junction where the ends of the arms are dramatically different will show a larger IDA, while a structure where the ends are nearly equally displaced from the center (as with ACmC-2Ca) will show a more shallow IDA.

In contrast, the ACBu-2Ca junction has a shallow \( J_{\text{twist}} \) (37.1°), but the largest IDA (68.5°) of any junction to date. This arises from the unusually narrow \( J_{\text{roll}} \) (130.4°) for the junction. In this case, the junction center becomes significantly displaced from the molecular center of mass towards the short 4 bp arms. Thus, the IDA, which relies on the junction rather than molecular center, becomes larger because the end of the shorter arm is pulled away from the vertex of the angle.

### DISCUSSION

With the implication of recombination-type processes in various cellular repair mechanisms, there is now renewed interest in the structure of the Holliday junction as a key intermediate in recombination. The recent single crystal structures, however, now make it possible to define the geometry of this complex in much greater detail and relate this to the effects of sequence. We describe here methods for explicit calculation of the geometric parameters \( J_{\text{roll}}, J_{\text{slide}}, \) and \( J_{\text{twist}} \) for DNA Holliday junctions that are based on the helical features of individual structures rather than relying on comparisons with a presumed reference conformation. These three parameters are given names that are conceptually analogous to those used to describe similar rotational and
translational relationships between linked and adjacent base pair steps along a DNA double helix. The two opposing stacked duplexes are treated as being analogous to the two base pairs of a dinucleotide (in other words, our axis of reference that is best analogous to the double helix axis would run parallel to the junction crossover and perpendicular to the dyad axis). Thus \( J_{\text{slide}} \) is the sliding of the two stacked duplexes relative to each other, just as helical slide is the sliding of two base pairs relative to each other, and so forth.

The global geometry of the junction in its native stacked-X form had previously been analyzed from a set of theoretical models (36,38), with a very explicit set of geometric parameters used in the analysis of all possible rotational relationships between the stacked duplex arms (38). It is immediately obvious that the parameters that we have defined here are analogous to these earlier definitions [our \( J_{\text{twist}} \) is analogous to the interduplex azimuthal angle of Srinivasan and Olson (38), and related to this angle by 180°]. However, a closer examination of the theoretical models presented in this report showed that this azimuthal angle is not consistent with the experimentally derived models. Thus, to maintain consistency among the experimental studies and to minimize confusion, it was important to develop this set of definitions.

The values of \( J_{\text{twist}}, J_{\text{slide}} \) and \( J_{\text{roll}} \) determined empirically without use of fitted helical axes are better correlated to analytical values determined using a global helix axis defined by the CURVES program for nucleic acid analysis (34) than to those using CURVES local axes or either local or approximate global axes defined by the alternative program 3DNA (35). In addition, the macroscopic IDA, which has been determined experimentally by gel electrophoresis, FRET and AFM measurements on immobilized junctions, is defined at the atomic level by the individual components \( J_{\text{roll}}, J_{\text{slide}} \) and \( J_{\text{twist}} \). At this point, we can start to correlate specific parameters with classes of junctions and their particular sets of intramolecular interactions as seen in single-crystal structures, and attempt to relate these to the conformation of the Holliday junction as determined by non-crystallographic means.

From the values of \( J_{\text{slide}}, J_{\text{roll}} \) and now \( J_{\text{twist}} \) explicitly determined for individual structures, the crystal structures of Holliday junctions can be directly compared with each other without the need for a reference structure (Table 2). As expected, the least distorted of the junctions in terms of \( J_{\text{slide}} \) are the sodium forms of the inverted repeat sequence d(CCGGTACCAGG) (ACC-4Na and ACC-2Na) which had previously served as references for comparison. These structures have \( J_{\text{slide}} \) values between \( -0.36 \) and \( +0.23 \) Å, indicating that the arms are translated only slightly towards either end relative to the junction crossover. For this set of structures, \( J_{\text{twist}} \) ranges from \( -38 \) to \( -40 \)°, while \( J_{\text{roll}} \) varies from 141 to 145°. Replacing the sodium with Ca²⁺ ions does not dramatically affect any of these parameters, nor does replacing the terminal C-G base pairs with T-A base pairs in the decanucleotide. The one exception is the tACC-2Ca structure, which has a \( J_{\text{slide}} > 1 \) Å, and a \( J_{\text{roll}} \) that is slightly more open to expose the major groove surfaces of the junction. On average, the junctions that are characterized with the central ACC core trinucleotide in the presence of Na⁺ or Ca²⁺ form a group with average \( J_{\text{twist}} = 38.9° \) (1.11° SD), average \( J_{\text{slide}} = 0.25 \) Å (0.46 Å SD) and average \( J_{\text{roll}} = 143.3° \) (2.14° SD). This set of structures are characterized by a set of well-defined intramolecular interactions that link the C₇ and C₈ cytosine bases of the A₀C₇C₈ trinucleotide core to the phosphate oxygens that cross between the stacked duplex arms across the junction. These interactions involve a direct hydrogen bond from the N4 nitrogen of C₈ to the C₇ nucleotide phosphate oxygen, a solvent-mediated hydrogen bond from G₃ to the A₀ nucleotide phosphate oxygen, and a hydrogen bond from the N4 nitrogen of C₇ to the A₀ nucleotide phosphate oxygen. As these interactions are disrupted or modified by sequence changes, base modification or addition of other ions, the geometry of the junction is also affected. For example, incorporating the larger Sr²⁺ cation into the structure opens the \( J_{\text{twist}} \) by 6° to an average of 45° (0.64° SD) and the \( J_{\text{roll}} \) by 8° to an average of 150.2° (0.14° SD). The duplexes are also slightly shifted to give an average \( J_{\text{slide}} \) of 0.58 Å.

The structure of GCC-2Na extended the core motif to include a guanine at position 6 of the now expanded Pu₄C₅Py₈ trinucleotide core. The geometry of GCC-2Na, where the adenine of the ACC core is replaced by a guanine base, is nearly identical to that of the ACC-containing junctions in terms of all three geometric parameters. Again, this is not surprising, since the two structures are completely superimposable at the atomic level (with an r.m.s.d. of 0.426 Å for identical atoms) (24), and nearly all of the intramolecular interactions observed in the ACC structures are also seen here. This reinforces the assertion that the purine at this sixth position of the trinucleotide core does not play as significant a role in defining the junction conformation as do the two cytosines of the core.

The gACC-2Na junction shows the largest \( J_{\text{roll}} \) (155.9°) and thus has the most accessible set of major groove surfaces at the junction. This significant opening of the junction to expose the major groove can be directly attributed to the unusual base pairing interactions associated with the G-A mismatched base pair at the junction crossover. This structure also shows the most distinct difference between the \( J_{\text{slide}} \) values as determined by the empirical versus the analytical methods, with the two general methods yielding \( J_{\text{slide}} \) values of opposite signs. This difference can be attributed to the slight differences in the position of the molecular center (used in the empirical calculations) relative to the positions where the helix axes intersect the bisecting plane (which are used for the analytical calculations).

The sequence motif for junctions was further extended with the structures of ACmC-2Ca and ACbU-2Ca, which showed that the trinucleotide core can accommodate cytosine methylation and, for the first time, a thymine-type nucleotide (in this case a 5-bromouracil) at position 8. More importantly, the structures demonstrated, as discussed previously and above in the Results, that these substituents induce conformational variability by significantly affecting the \( J_{\text{slide}} \) and/or \( J_{\text{roll}} \) of the junction duplex arms. In addition, we see here that the ACbU-2Ca structure is more perturbed than originally thought.

We had previously described the ACbU-2Ca structure as being surprisingly unperturbed, given the presence of a large bromine atom at the junction crossover and loss of several atomic interactions postulated to be important for junction core stability (11,24). The current study indicates that, much to the contrary, the ACbU-2Ca structure is one of the most
rotationally perturbed Holliday junction structures to date, with the largest IDA that results from a large \( J_{\text{roll}} \) despite having one of the smallest \( J_{\text{twist}} \) values. In addition, our previous analysis indicated that this structure had little or no \( J_{\text{slide}} \), but we observe here a significant (0.75 Å) sliding of the duplexes. The discrepancy between the two conclusions concerning the degree of perturbation lies primarily in how \( J_{\text{roll}} \) was determined in the earlier study. Our previous calculation of \( J_{\text{roll}} \) relied on defining vectors from the junction center to the outside of the helix cylinder according to the positions of individual phosphorus atoms on the outside strands. In ideal cases, where the base pairs around the junction crossover are standard B-DNA, this calculation is reasonably representative of the rotational relationship between the stacked duplexes. However, in the case of ACbU-2Ca, these base pairs are significantly buckled. These distortions are local effects that shift the phosphates from the ideal B-DNA positions but, when used to define vectors, greatly distorted the apparent global features of the duplex, which \( J_{\text{roll}} \) is intended to measure. Likewise, \( J_{\text{slide}} \) values for junctions are fairly comparable when the duplex arms of the junctions have similar values for rise per base pair. However, in the case of ACbU-2Ca, there is on average a 0.1 Å difference in rise per base pair between this structure and ACC-4Na which masks the true translation of the entire duplex along its helix axis; thus a structure that was previously thought to have little or no \( J_{\text{slide}} \) actually has a significant amount.

One of the unresolved issues concerning the single-crystal structures is that the IDAs of \(-41°\) are significantly more shallow than the \( 60° \) that has been seen from non-crystallographic studies on immobilized junctions. It is now apparent that the definitions of the IDA were used inconsistently in this comparison. We see that the shallow angle reported from the crystal structures was actually a measure of \( J_{\text{twist}} \) rather than the end to center to end angle that is determined by, for example, FRET measurements (27–29). The actual IDA determined for the single-crystal structures (average IDA = 62.3°, 3.13° SD) would appear to put this controversy to rest, but does it? There is a clear relationship between IDA and \( J_{\text{twist}} \) as the duplex arms become longer, the IDA approaches the values for \( J_{\text{twist}} \) (Fig. 4), since the contribution of the width of the B-DNA double helices and the contributions of \( J_{\text{slide}} \) and \( J_{\text{roll}} \) to the IDA become negligible. For junctions with short arms, however, such as the sequences of the single-crystal structures, these conformational components cannot be ignored. When the \( J_{\text{twist}} \) values of \(-41°\) of the crystal structures are extrapolated to the longer arms of the constructs used in the non-crystallographic studies, the resulting IDAs predicted for the junction in solution are generally only 2–3° greater than the \( J_{\text{twist}} \) value of \(-45°\), not \( 60° \) as was measured. Thus, there remains significant discrepancy between the relationships of the stacked arms in the crystal structures and in the constructs used in solution studies. The evidence points toward sequence-dependent interactions between the major groove surface of the stacked duplex arms with the crossover phosphates. For example, AFM studies (31) on sequences analogous to those used in the FRET and gel studies (25, 29, 31, 32) measure IDAs of \(-60°\), while AFM on sequences that include the ACC trinucleotide core of the initial two junction structures (ACC-4Na and gACC-2Na) resulted in an IDA of \(-41°\) (33). We should note that this latter value is more consistent with a \( J_{\text{twist}} \) value because of how the sheets of junctions lie on the mica surface, which is analogous to the crystallographic resolving plane. Therefore, the resulting angle is directly comparable with the \( 41° \) \( J_{\text{twist}} \) values seen in the crystal structures, indicating, as previously proposed, that the trinucleotide core and the intramolecular interactions associated with this sequence motif are primarily responsible for defining the geometric features of the Holliday junction.

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