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ABSTRACT

We describe here a method using differential scanning calorimetry (DSC) to determine the energy of biomolecular halogen bonds (X-bonds) in a DNA system. This system is unique because we are able to isolate the energetics of a specific molecular interaction from the thermodynamic properties of an entire system. To achieve this goal, we engineered DNA Holliday junctions that are stabilized by either an X-bond or a hydrogen bond (H-bond); the thermodynamic stability of these Holliday junctions are measured through DSC melting studies. Stabilizing energies of the competing X- and H-bonds were determined outside of all of the other interactions by subtracting the energies of these junctions. Overall, the X-bond was shown to have a greater stabilizing potential than the H-bond in this DNA junction system.

BACKGROUND

Differential scanning calorimetry (DSC) measures the change in heat capacity for thermal melting, from which we can derive the melting temperature (T_m), change in enthalpy (ΔH), and change in entropy (ΔS) for the system. The only limitation of this technique is that these values are measured for the melting of an entire system. The question is how can DSC be used to determine the energies of a specific molecular interaction? We describe here how an experimental system was designed to specifically determine the thermodynamic stability of a single halogen bond (X-bond) in a biological environment.

An X-bond is a stabilizing electrostatic interaction between the electropositive crown of a polarized halogen (F, Cl, Br, I) and an electron-rich nucleophile, in which the distance between the two interacting atoms are less than the sum of

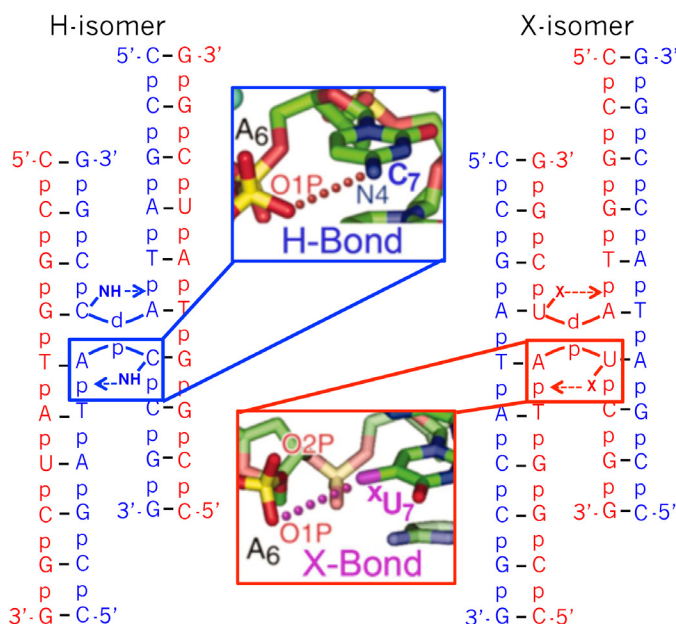


Figure 2. Holliday junction construct competing a halogen bond (X-bond) against a hydrogen bond (H-bond): Schematic of the H-bond stabilized junction (H-isomer) and the X-bond stabilized junction (X-isomer). Highlighted are the structures of the H and X-bonds at the crossover region of the respective junctions.

their standard van der Waals radii ($\sum r_{vdW}$) [1], Figure 1. The origin of the positive crown of the halogen is best described by the σ -hole model [2] (reviewed in [1, 3]), and follows the trend in which larger halogens are associated with stronger polarization ($I > Br > Cl > F$). For the studies described here, we constructed DNA Holliday junctions to measure and compare the energies of the strong X-bonds formed by bromine and iodine.

Crystal structures of the DNA junctions used in this study show that they are stabilized by a hydrogen bond (H-bond) [4-6] from the N4 amino group of the C₇ cytosine base to a phosphate oxygen on the backbone of the DNA (Figure 2). Previous studies have shown that cytosine can be replaced by a halogenated uracil, which results in the replacement of the stabilizing H-bond with an X-bond [7] (Figure 2). With careful data analysis, this unique experimental system allows us to compare and contrast the stabilizing energy of an H-bond against that of an X-bond in a biomolecular context.

In order to use DSC to monitor a specific molecular interaction, we must be able to deconvolute the melting curve data of an entire system into its components parts. Thus, we must first subtract out all the other contributing

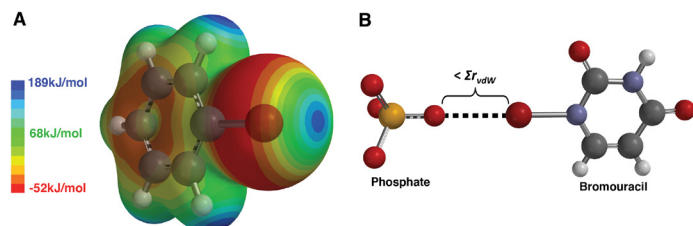


Figure 1. Halogen bonding: A. Bromobenzene molecule overlaid with its electrostatic potential map, calculated at the MP2 level. The polarization of the bromine results in an electropositive crown (blue) and an electronegative belt (red) around the halogen atom. B. Halogen bonding (X-bonding) is evidenced by a distance between interacting atoms that is shorter than the sum of their van der Waals radii ($\sum r_{vdW}$). In this example, the bromine of a 5-bromouracil is interacting with a negatively charged oxygen of a phosphate group.

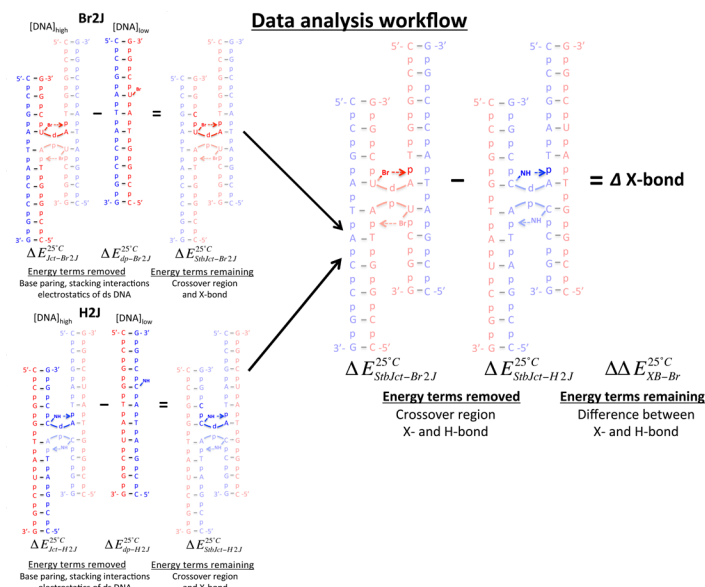


Figure 3. Data analysis flowchart: Schematic of how to process the data to get the relative energies of stabilization for X-bonds versus H-bonds. Standard thermodynamic relationships are used to extrapolate the energies to a reference temperature (25°C). Next, the stabilizing energy of the junction ($\Delta E_{\text{StbJct}}^{25^\circ\text{C}}$) is found by subtracting the duplex energies from the junction energies. The relative energies of the X-bond ($\Delta E_{\text{XB}}^{25^\circ\text{C}}$) are found by subtracting the energy of the halogenated junctions from the non-halogenated junction. This schematic illustrates the data analysis for the Br2J structure; this process is then applied to the I2J data. ΔE denotes ΔH , ΔS , or ΔG .

concentrations as duplex DNA and, by subtracting the low from the high concentration energy data, we can account for molecular interactions such as base stacking, H-bonding, and steric effects that are common among the two structural forms. What remains, then, are the energies associated with only the molecular interactions at the DNA junction. Now, by comparing the DSC energies of two different DNA junction constructs, one forming the interaction of interest (in this case, what we call the X2J construct that is stabilized by two X-bonds) and one without this interaction (an H2J construct that is stabilized by two Hbonds), we can subtract out all the other interactions within the junction, and focus only on the contribution of the X-bond versus the H-bond to the stability of the four-stranded complex (Figure 3).

METHODS

Oligonucleotide Purification and Assembly

Chemically synthesized DNA oligonucleotides were obtained from Midland Certified Reagent Company on the solid controlled-pore glass (CPG) with the dimethoxytrityl (DMT) protecting group still attached. Oligonucleotides were purified by reversed-phase HPLC followed by size exclusion chromatography on a Sephadex G-25 column after detritylation. Oligonucleotide sequences for this study are listed in Table 1. DNA oligonucleotides of each sequence were mixed in equimolar concentrations, varying from $15\mu\text{M}$ to $300\mu\text{M}$, in 1mM calcium chloride and 50mM sodium cacodylate at pH 7.0. The solutions were heated to 90°C for one hour and then slowly cooled to room temperature overnight before melting studies were conducted.

Table 1. Sequences DNA constructs to study X- and H-bonds in Junction. Strands of each sequence are mixed in equal molar concentrations, and annealed prior to DSC studies. BrU and IU are the nucleotides 5-bromouracil and 5-iodouracil, respectively.

Construct	Complementary sequences	Junction stabilized by
H2J	5'-CCGGTAUCGG-3'/5'-CCGATACCGG-3'	H-bond
Br2J	5'-CCGGTA(BrU)CGG-3'/5'-CCGATACCGG-3'	X-bond
I2J	5'-CCGGTA(IU)CGG-3'/5'-CCGATACCGG-3'	X-bond

DSC Melting Studies

Melting curves of the DNA at each concentration were measured using a TA Instrument Nano DSC held at a constant pressure of 3.0 atm . All samples were run against matching buffer conditions in the reference cell, with heating cycles from 0°C to 90°C at a scanning rate of $1^\circ\text{C}/\text{min}$, preceded by an initial equilibration time of 900 s . Experiments were repeated at least three times. Melting curves were measured for each construct at multiple concentrations of DNA to compare the sequences as fourstranded junctions and as duplexes. Each melting curve was analyzed using the NanoAnalyse software from TA Instruments (NanoAnalyse Data Analysis, version 2.3.6) to extract the enthalpy of melting and the melting temperature for each component in the sample. The best fit was determined by examining the standard deviation of the fit to the data and average weight term (AW). An $\text{AW} \approx 1.0$ indicates that the concentration of the sample is consistent with the model used to fit the data.

RESULTS

Deriving Thermodynamic Properties

The DNA constructs used for these studies have been previously shown to form duplex at low concentration [5] ($15\text{--}20\mu\text{M}$) and the DSC scans at these concentrations are primarily associated with melting duplex into single-stranded DNA. The types of energies that contribute to this melting event include base pairing, base stacking, and any additional effects related to base modifications (such as the halogenated uracil). Data at the lower DNA concentration were best fit using a single-component, two-state model (Figure 4A). The low DNA concentration scans all had fitted AW values near 1.0 (Figure 4D).

Melting curves of constructs at high DNA concentrations ($100\text{--}300\mu\text{M}$) showed T_m values shifted to higher temperatures relative to the values seen for the same constructs at lower concentrations. In addition, the enthalpy of melting is about 100% higher at high concentrations as compared to the low. When analyzing the data with a single component two-state model, as with the curves at low DNA concentrations, the AW term approached 2 (Figure 4B), indicating that there was twice the mass of DNA in the system than what was entered into the analysis. This result can be explained by assigning the DNA to a four-stranded junction form, which has twice the mass of the duplex DNA and therefore, the molar concentration is half relative to the duplex DNA. In addition, the one-component model gave a poor fit with large residuals, which lead us to analyze the data using

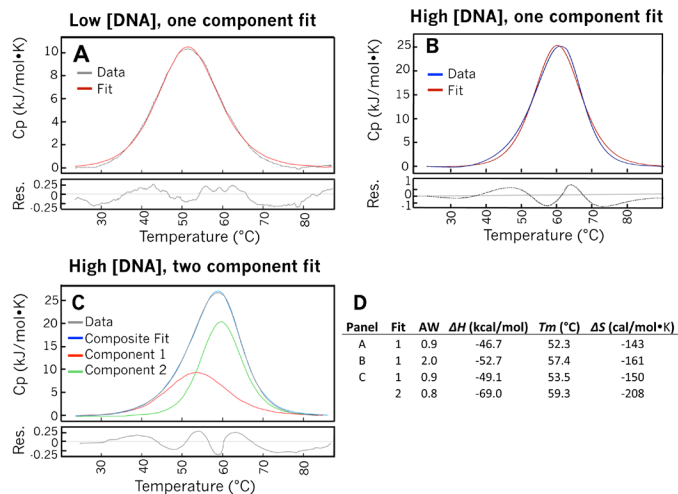


Figure 4. Differential scanning calorimetry melting curves and thermodynamic parameters of I2J: Melting curve data of the low DNA concentrations (A) and high DNA concentrations (B) of I2J, each fit with a single component two-state model. Melting curve of high DNA concentration (C) data refitted with a two component, two-state model. The composite of the two components is shown in blue. Residuals are shown beneath each fit. The thermodynamic parameters of each fit are listed in panel D, with the stabilization enthalpy (ΔH_m) and entropy (ΔS_m) at the melting temperature (T_m).

a two component, two-state model (Figure 4C). The two components of the model are distinct for each curve, with the first showing T_m and ΔH values similar to the duplex DNA parameters determined at low concentration. Therefore, we assigned this first component to the melting of the DNA in its duplex form. The second component has a higher T_m and ΔH , and this was assigned to the melting of DNA in its junction form into singlestranded DNA. The resulting AW terms using the two-component fit approach the value of 1.0, indicating that this model fits the data better than the single component model. The reported values for ΔH and ΔS at the melting temperature were taken as the averages of all measurements for each construct at their respective low and high concentrations. The melting energies (ΔH and ΔS at T_m) relate to the stabilization energies (enthalpy ΔH_m and entropy ΔS_m at the T_m), which are equal and of the opposite sign to the melting energies.

Determining the Specific Interaction Energy of an X-bond Relative to an H-bond

Once the stabilizing energies have been calculated at the T_m for all the constructs, the energy values were extrapolated to a reference temperature (25°C) so that they can be directly compared. This is done with standard thermodynamic equations (Figure 5A, B). To extract the energy of the X-bond in reference to the H-bond, the other contributing interaction must be subtracted from the overall thermodynamics of the system. Figure 3 is a flowchart on how this procedure is performed. Stabilization energies of the nucleotides at the junction crossover ($\Delta H_{StbJct}^{25^\circ C}$, $\Delta S_{StbJct}^{25^\circ C}$, $\Delta G_{StbJct}^{25^\circ C}$) are determined by subtracting the duplex energies from the junction energies (Figure 5C). This subtraction removes interaction energies like H-bonds, stacking interactions, and electrostatic effects of the double stranded arms of the DNA from the data. Subtracting the stabilization energy of the junction for an

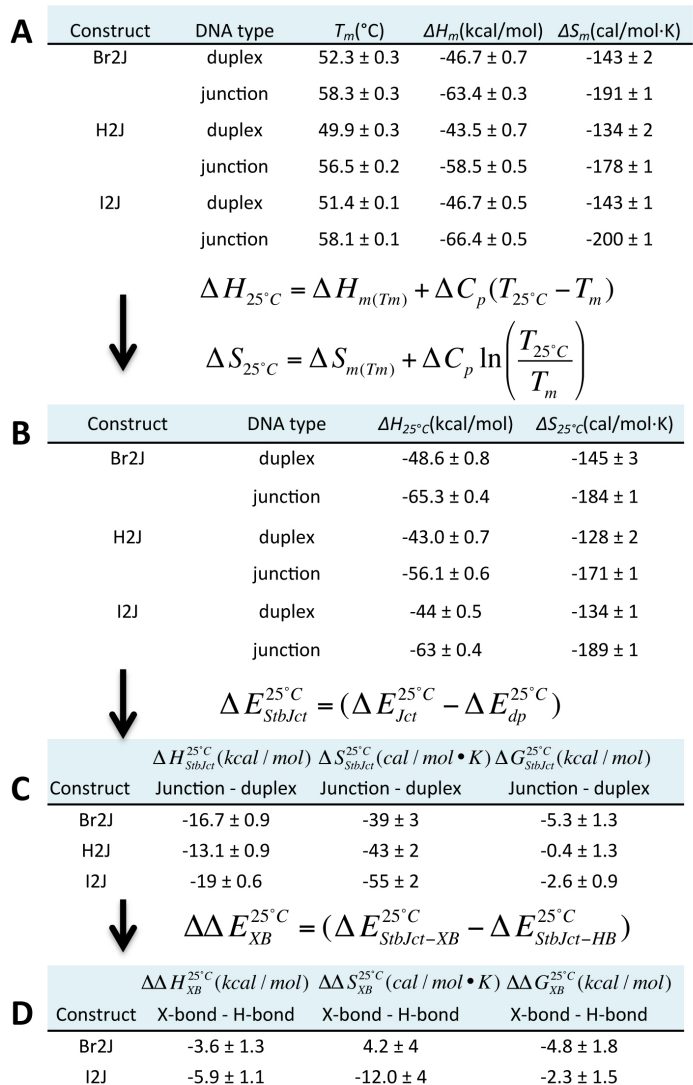


Figure 5. Thermodynamic parameters for the stabilization of DNA junctions. (A) Melting enthalpies and melting temperatures for each construct were measured by DSC, and then converted to stabilizing enthalpies and entropies. (B) Energies were extrapolated to a reference temperature (25°C) via standard thermodynamic equations. (C) The difference in thermodynamic parameters for the junction and duplex results in the stabilizing energies associated with crossover region of the junction for the H2J, Br2J, and I2J structures ($\Delta H_{StbJct}^{25^\circ C}$, $\Delta S_{StbJct}^{25^\circ C}$, $\Delta G_{StbJct}^{25^\circ C}$). (D) The energies of the X-bond relative to the H-bond ($\Delta \Delta H_{XB}^{25^\circ C}$, $\Delta \Delta S_{XB}^{25^\circ C}$, $\Delta \Delta G_{XB}^{25^\circ C}$) are calculated by subtracting the respective junction minus duplex terms for the Br2J or I2J junctions from the H2J junction. ΔE denotes ΔH , ΔS , or ΔG .

X-bond ($\Delta H_{StbJct-XB}^{25^\circ C}$, $\Delta S_{StbJct-XB}^{25^\circ C}$, $\Delta G_{StbJct-XB}^{25^\circ C}$) from the stabilization energy of the junction for an H-bond ($\Delta H_{StbJct-HB}^{25^\circ C}$, $\Delta S_{StbJct-HB}^{25^\circ C}$, $\Delta G_{StbJct-HB}^{25^\circ C}$) will remove all the other molecular interactions associated with the crossover region of the junction. What remains is the specific enthalpy, entropy, and Gibbs's free energy ($\Delta \Delta H_{XB}^{25^\circ C}$, $\Delta \Delta S_{XB}^{25^\circ C}$, $\Delta \Delta G_{XB}^{25^\circ C}$) of an X-bond relative to an H-bond for the bromine and iodine X-bonds in the Br2J and I2J structures respectively. (Figure 5D).

DISCUSSION AND CONCLUSIONS

The initial energies measured at the melting temperature show that the Br2J and I2J constructs have higher T_m and

ΔH_m than the H2J construct (Figure 5A). The T_m and ΔH_m for the X2J structures increased by about 2°C and 2 kcal/mol respectively. An increase in T_m and ΔH_m is expected if a stabilizing interaction is introduced into a system. In order to directly compare the energies of each construct, the energies were extrapolated to the standard reference temperature (25°C). At this temperature the Br2J and I2J structures were found to have larger enthalpy values than the H2J (Figure 5B) ($\Delta H_{25^\circ\text{C}}$), which indicates that the halogenated structures are more stable. The stabilizing energies of junctions ($\Delta H_{25^\circ\text{C}}^{\text{StbJct}}$, $\Delta S_{25^\circ\text{C}}^{\text{StbJct}}$, $\Delta G_{25^\circ\text{C}}^{\text{StbJct}}$) were determined for all three constructs (Figure 5C) by subtracting duplex energies from the junction energies. This subtracts out the energies of those interactions that are common to both the junction and duplex forms of the DNA (for example: base pairing and stacking interactions, and electrostatic effects of the DNA backbone). The data, now focusing only on the structural features at the junction crossover, show that the X-bond stabilized junctions (Br2J and I2J) were more favorable in terms of enthalpy and free energy ($\Delta\Delta H_{\text{XB}}^{25^\circ\text{C}}$, $\Delta\Delta G_{\text{XB}}^{25^\circ\text{C}}$) than the H-bond stabilized junction. Furthermore, the iodine X-bond was more enthalpically favorable than the bromine (Figure 5D). This result can be explained by the increased polarization of the iodine relative to bromine, as predicted by the σ -hole model. However, the increase in enthalpy comes at an entropic cost, with the iodine X-bond having a decrease in ΔS and the bromine X-bond being associated with a slight increase in ΔS . The decrease in entropy for the I2J structure can be attributed to fitting the larger iodine into a tight space, which makes the junction more rigid. Consequently, the bromine X-bond has a more favorable overall free energy of stabilization than the iodine X-bond in this system. These data show that there is a balance between the entropic and enthalpic terms of the free energy that results in the bromine forming the optimum X-bond for this system. Overall, the X-bond was able to add 2-5 kcal/mol more stability to this system compared to the H-bond.

Knowing the amount of energy that an X-bond can contribute to the stability of a system relative to an H-bond is useful from a biomolecular engineering point of view. For example, a scientist wants to add about 2-5 kcal/mol more stability to a system without adding additional interactions, this could be achieved by replacing an H-bond in the system with an X-bond. This type of substitution can also be used in hit-to-lead drug development where an X-bond could replace a H-bond, which in turn would increase the stability of the drug into its binding pocket. Increased stability is generally associated with stronger binding affinity.

Overall, this study used the change in heat capacity for the thermal melting of an entire system, and was able to determine the specific thermodynamic properties of single chemical interaction. This was done by carefully designing our experimental system so that we could subtract out all the other contributing interactions, thus, only leaving the X-bond energy.

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