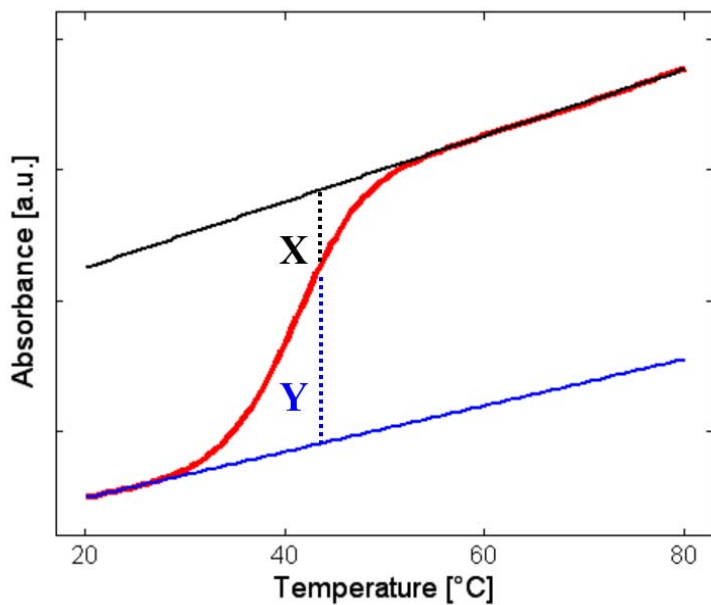
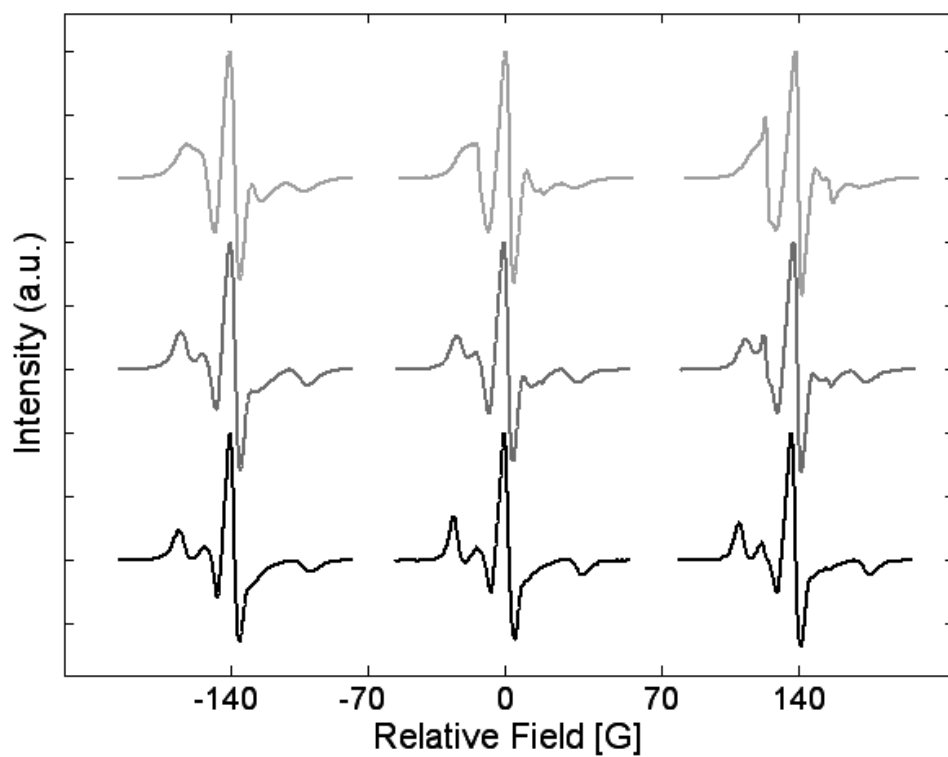


## Supporting Information



**Figure S1:** Example of melting curve (red), from sample GÇ/T. The black line represents the linear extrapolation of the melted (single-stranded) state. The blue line represents the extrapolation of the unmelted (double-stranded) state.



**Figure S2:** EPR spectra from a simulated spectra of a 14-mer with no internal motion (left column), samples of control I (middle column), and IV (right column) of Figure 3 at 40 °C (light grey), 20°C (medium gray), and 0 °C (black) .

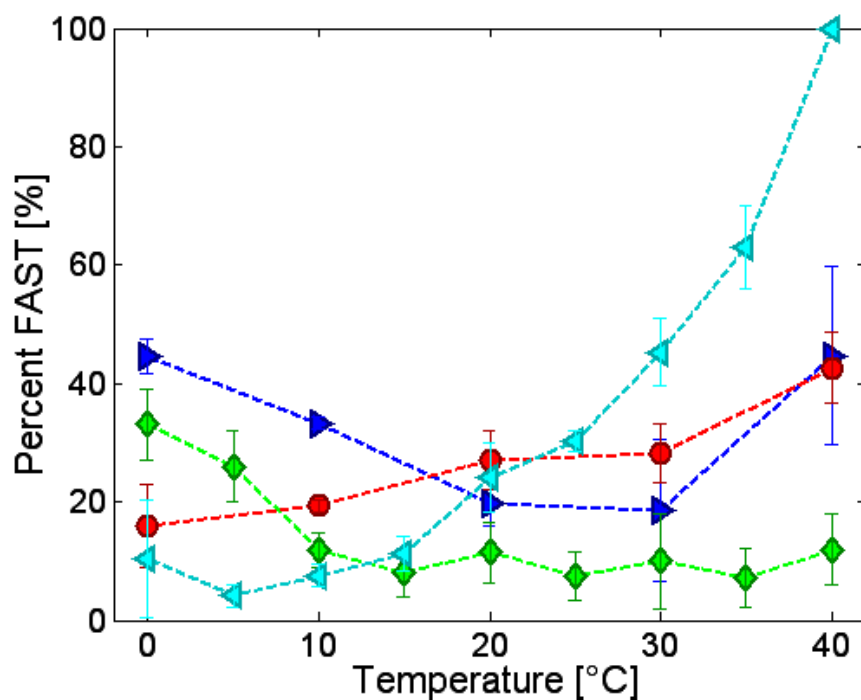
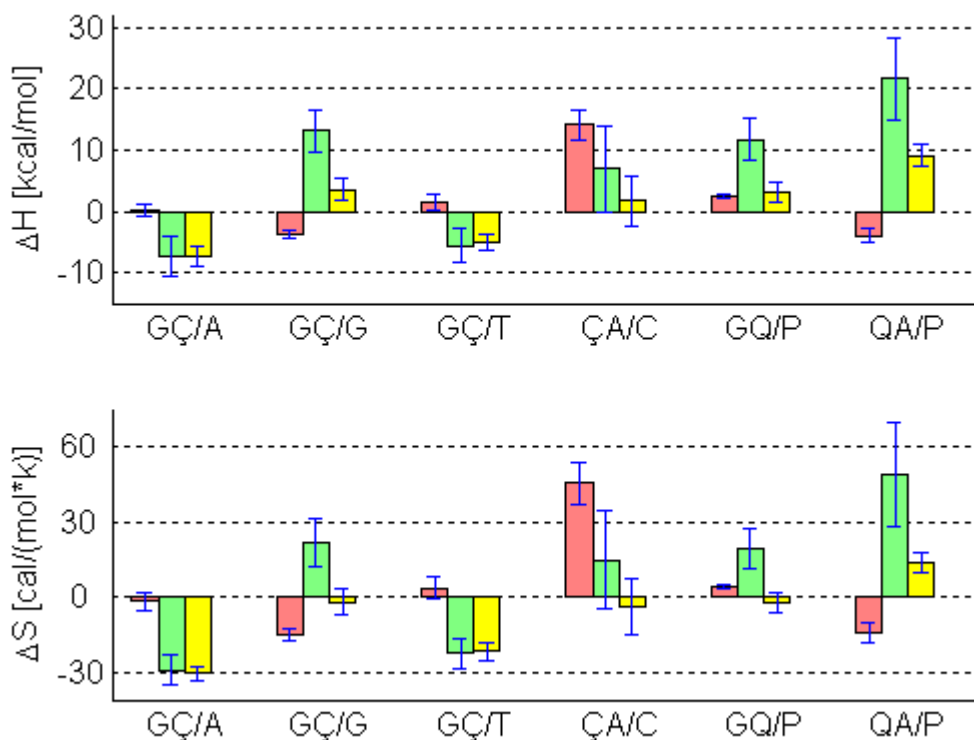


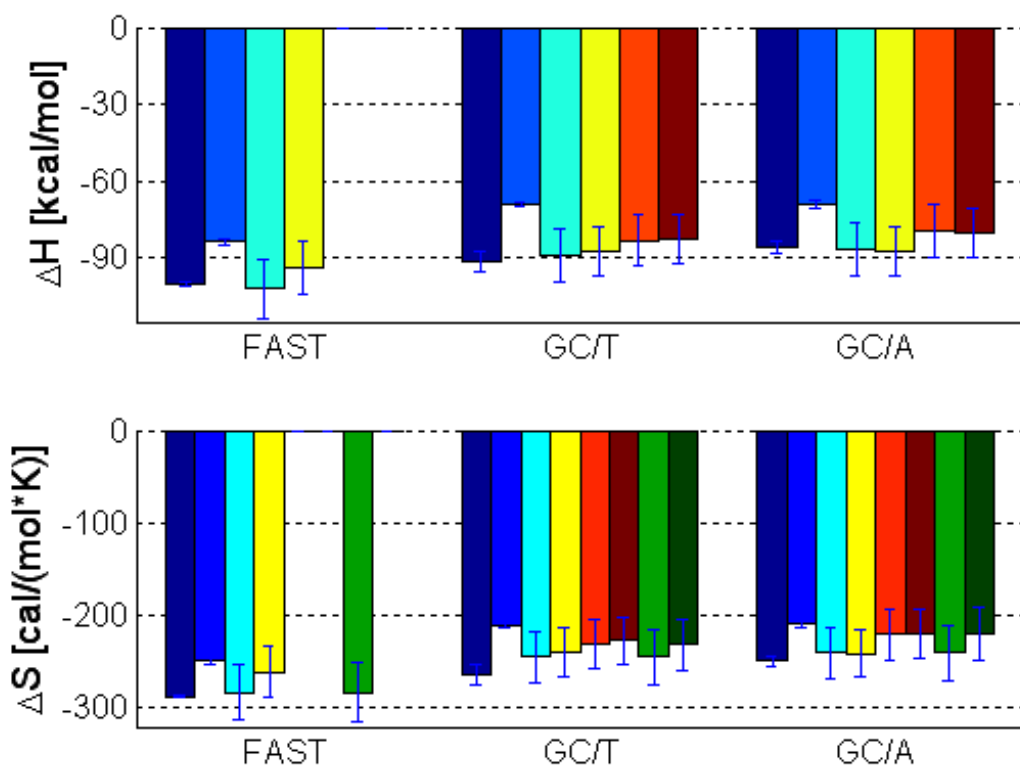
Figure S3: Results of fitting spectra to a sum of SLOW spectra and FAST spectra, but here the SLOW control is considered to be the “closest match” Ç/X sample. Both controls are at the same temperature as the test sample spectrum. GÇ/A (blue right-pointing triangles) with Ç/A used as a SLOW reference, GÇ/T (red circles) with Ç/T used as the SLOW reference, GÇ/G (green diamonds) with Ç/G used as a SLOW reference, and ÇA/C (cyan left-pointing triangles) with Ç/C used as the SLOW reference (as in Figure S3). Dotted lines are added only to aid the eye.



**Figure S4:**  $\Delta H_D$  and  $\Delta S_D$  for the interconversion from SLOW→FAST. Shown are the values derived from the EPR spectral fitting and the van't Hoff equation (1.3) (pink), the NN calculations with the intervening basepair included (1.5) (green), and the NN calculation without the intervening basepair (1.6) (yellow).

***Calculation of Thermodynamics of Formation: Enthalpies and Entropies of Formation Calculated from the Unified NN Model and Parameters***

An attempt was made to compare DSC or UV melting results to the NN values, by calculating the thermodynamics of formation with the NN parameters. Thermodynamic parameters were estimated using the NN model following Equations (1.5) and (1.6), but instead of solving for one value, the difference between the  $\sum_i n_i \Delta H_f(i)$  values in the SLOW and FAST conformations, we report a separate  $\sum \Delta H_{f,NN}$  for the SLOW or FAST conformation, twice for each conformation: once including the intervening basepair, as in (1.5), and then again ignoring the adjoining basepair, as in (1.6). In the NN model, the value of the enthalpy for a one base bulge was assumed to be negligible; therefore, there is no separate NN calculation for the FAST or SLOW conformations with the bulge included in the calculation.<sup>32</sup>



**Figure S5: Comparisons of experimental data and NN-based calculations for  $\Delta H_f$  and  $\Delta S_f$ .**

Subscripts indicate whether the calculation was assuming a SLOW or FAST conformation, and whether or not the intervening basepair was included (INT denotes inclusion). The values derived from the UV-monitored melting curves (navy blue), the DSC experimental results (royal blue), NN<sub>FAST</sub> (light blue), NN<sub>FAST-INT</sub> (yellow), NN<sub>SLOW</sub> (orange), NN<sub>SLOW-INT</sub> (brick red). For the entropy calculation only, NN<sub>FAST-BULGE</sub> (light green) and NN<sub>SLOW-BULGE</sub> (dark green) conformation. Only FAST conformation calculations (NN<sub>FAST</sub>, NN<sub>FAST-INT</sub>, and NN<sub>FAST-BULGE</sub>) are given for the FAST control, as it never exists in the SLOW conformation.

The thermodynamic parameters for the melting of the FAST control, **GC/T** and **GC/A**, are compared with different predictions of the NN model (**Figure S5**). Reasonable agreement is seen between these results and the NN model, within the errors of the NN model parameters. However, the experimental results from UV monitored melting and the DSC experiments are distinct, even when considering the errors. This further underscores the difficulty of applying the two-state model. For example, when comparing the NN model predictions for the **GC/T** construct against the UV monitored melting data, the NN model suggests that a **G:T** pair (FAST conformation) is the dominant configuration in the pre-melting region. However, when compared against the DSC data, the NN model predicts a **C:T** pairing (SLOW conformation) as the dominant conformation. The errors associated with the NN model make it difficult to draw any strong distinction.

## EPR Studies Examining the Distance and Orientation Between Two Spin Labels of a Bulged Sample

Structures of samples labeled with two spin labels are shown in **Figure S6** in **Supporting Information**. The results for [1] and [2] show the large changes spectra undergo due to spin-spin dipolar coupling, giving a calculated distance of 7 Å between spin probes for [1].<sup>48</sup> The spectra in **Figure S6** are scaled only to clearly show the lines. The singly labeled duplex **3** is shown for two reasons: First, there is a singly labeled contaminant (of about 8%) present in the spectra of the two dipolar-interacting spin-labels in close proximity **1**. The contaminant could not be removed and occurred in repeated syntheses. Second, the single label contaminant signal can be readily subtracted from the doubly-labeled spectra, and the dipolar broadened spectra prior to simulating the spectra to calculate a distance between spin labels.

The lineshape broadening shown in **Figure S6** [2], when there are two neighboring spin probes but one is bulged out, shows that the spins are much farther apart than in [1]. Examination of the low-field peak in the first two spectra of **Figure S6** shows a common broadening not seen in the singly-labeled spectra [3]. In the spectra for the bulged probe, [2], the two probes are about 11 Å apart. This conclusion is based on doubly-labeled DNA duplex experiments and using a well-established theory to calculate spin-spin distances.<sup>38,48</sup>

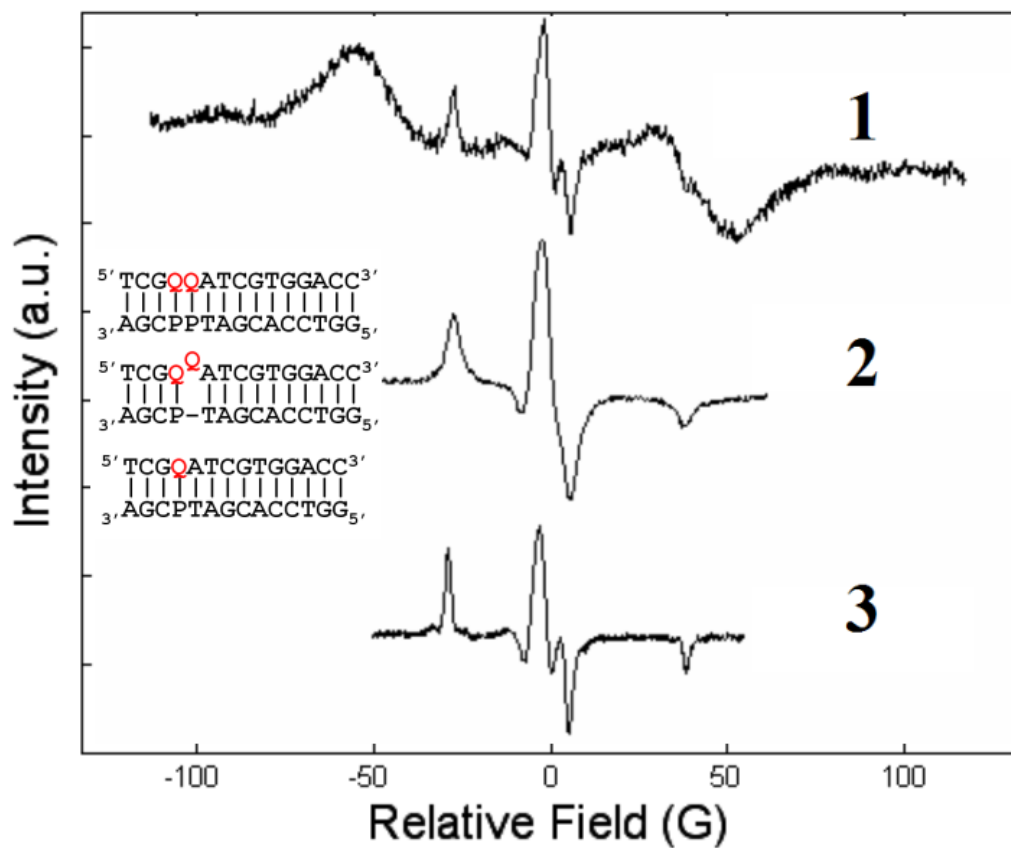


Figure S6: EPR spectra of 1) the duplex with two neighboring spin labels (top structure of inset), 2) the doubly labeled duplex with one probe bulged and one within the duplex (middle structure), and 3) the singly-labeled duplex (bottom structure), for comparison. Structures shown in inset, the spin probe Q is shown in red, and its proper basepairing partner, 2-aminopurine, indicated by P. All spectra at 0 °C in 50% sucrose/PNE w/v, pH 7.0.