An Isothermal Titration and Differential Scanning Calorimetry Study of the G-Quadruplex DNA-Insulin Interaction

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · JANUARY 2014

Impact Factor: 3.3 · DOI: 10.1021/jp411293r · Source: PubMed

THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

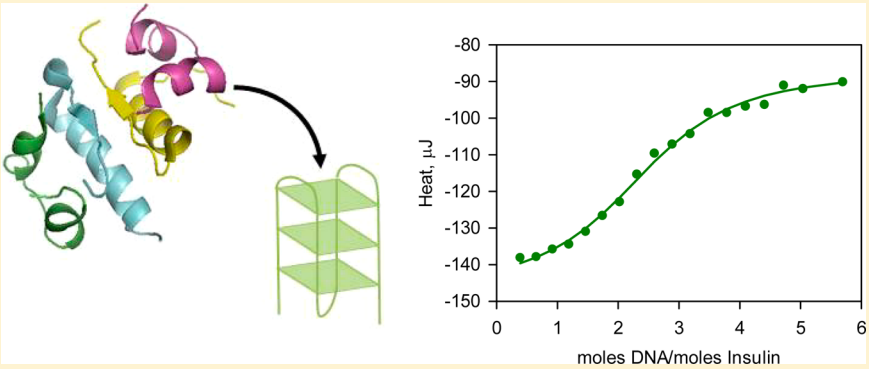
An Isothermal Titration and Differential Scanning Calorimetry Study of the G-Quadruplex DNA−Insulin Interaction

Christine M. Timmer,[†] Nicole L. Michmerhuizen,[†] Amanda B. Witte,[†] Margaret Van Winkle,[†] Dejian Zhou,[‡] and Kumar Sinniah*[,†]

[†]Department of Chemistry and Biochemistry, Calvin College, 3201 Burton Street Southeast, Grand Rapids, Michigan 49546, United States

[‡]School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Supporting Information



ABSTRACT: The binding of insulin to the G-quadruplexes formed by the consensus sequence of the insulin-linked polymorphic region (ILPR) was investigated with differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). The thermal denaturation temperature of insulin was increased by almost 4 °C upon binding to ILPR G-quadruplex DNA as determined by DSC. The thermodynamic parameters (K_D, ΔH, ΔG, and ΔS) of the insulin−G-quadruplex complex were further investigated by temperature-dependent ITC measurement over the range of 10−37 °C. The binding of insulin to the ILPR consensus sequence displays micromolar affinity in phosphate buffer at pH 7.4, which is mainly driven by entropic factors below 25 °C but by enthalpic terms above 30 °C. The interaction was also examined in several different buffers, and results showed that the observed ΔH is dependent on the ionization enthalpy of the buffer used. This indicates proton release upon the binding of G-quadruplex DNA to insulin. Additionally, the large negative change in heat capacity for this interaction may be associated with the dominant hydrophobicity of the amino acid sequence of insulin's β subunit, which is known to bind to the ILPR G-quadruplex DNA.

INTRODUCTION

Guanine-rich DNA sequences are prevalent in biologically significant regions of the human genome, including gene promoter and telomere regions.[1] Of particular interest is the propensity of guanine nucleotides to form Hoogsteen hydrogen bonds with one another, resulting in planar quartet structures. In the presence of cations, most notably potassium and sodium, these quartets stack one on top of each other to form guanine quadruplex (G-quadruplex) structures. The resulting helical structures can adopt a variety of stable topologies and have been identified and characterized *in vitro*.[1] More than 376000 sites in the human genome have been identified as having the potential to form G-quadruplexes,[2] which suggests that the biological functions of many of these structures have yet to be discovered. The rapid formation of G-quadruplexes *in vitro* suggests that the structures have the potential to provide kinetic accessibility to control gene activity and affect biological processes.[3] The existence of these structures in live cancer cells has recently been demonstrated by quantitative visualization.[4] This evidence of G-quadruplex formation has significant implications for the regulation of gene expression, as these structures could contribute to the stability or instability of genes and affect transcription.[5]

The biological role of these structures has been explored recently by examining the interaction of various proteins with G-quadruplex DNAs. A number of aptamers consisting of G-quadruplex-forming sequences have been shown to recognize protein targets with binding affinities in the submicromolar to nanomolar range.[1,2,6] Proteins, including thrombin, MyoD, Ku protein, and others, have been shown to bind to their respective G-quadruplex DNA structures with a stabilizing effect.[2a] Other helicase proteins have been shown to unwind G-quadruplex

Received: November 17, 2013
Revised: January 19, 2014
Published: January 24, 2014

   dx.doi.org/10.1021/jp411293r | J. Phys. Chem. B 2014, 118, 1784−1790

structures, and certain nucleases also cut DNA near or within G-quadruplexes.[2a] Taken together, these various studies have provided insight into the dynamic and significant roles these structures might play in biological processes.

The insulin-linked polymorphic region (ILPR) is a DNA sequence located 363 bp upstream of the human insulin gene. The ILPR contains multiple consecutive guanine nucleotides in tandem repeats of the consensus sequence 5′-$ACAG_4TGTG_4$-3′ that allow it to form G-quadruplex structures *in vitro*.[7] Type 1 diabetes has been recognized in humans with 30−60 repeats in this region, while individuals with 120−170 repeats are less susceptible to this condition.[8] Previous work has shown that an increased number of ILPR repeat sequences results in a higher transcriptional activity using transfected β cells.[9] While the association between genetic susceptibility to type 1 diabetes and transcription of the insulin gene is not yet well understood, the mechanisms that control ILPR G-quadruplex function could hold contributing factors.[6] Recently, insulin was found to bind to the G-quadruplex DNA structures formed by the ILPR sequence *in vitro* using affinity matrix-assisted laser desorption ionization mass spectrometry,[10] surface plasmon resonance (SPR),[10] fluorescence quenching,[6] and electrochemical detection.[11] SPR measurements of the consensus sequence (ILPR repeat a) showed a binding affinity (K_D) of 0.2−0.7 μM in phosphate buffer at micromolar concentrations of insulin, and a K_D of 0.17 μM in Tris-HCl buffer was obtained from the fluorescence quenching study.[6,10] These studies demonstrate a moderate binding affinity of insulin for the *in vitro* G-quadruplex DNA of the ILPR. This binding affinity suggests that the ILPR may be able to regulate the insulin concentration *in vivo* and consequently that insulin may play a role in regulating the transcription of its own gene.[5]

We report herein a complete thermodynamic characterization of the *in vitro* binding interaction between insulin and the G-quadruplex formed from the ILPR consensus DNA sequence by differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). DSC was used to demonstrate insulin stability upon binding to G-quadruplex DNA, while ITC was used to determine the dissociation constants (K_D) and thermodynamic parameters (ΔH, ΔG, and ΔS) at temperatures ranging from 10 to 37 °C. Furthermore, examining the binding interaction in different buffers revealed that the measured ΔH is dependent on the ionization enthalpy of the buffer with transfer of a proton from the insulin−DNA complex to the solvent. The change in heat capacity was also used to analyze the hydrophobic and vibrational components of the binding interaction.

MATERIALS AND METHODS

Materials. Potassium chloride (99.0%), Trizma hydrochloride (≥99%), phosphate buffer (1.0 M, pH 7.4 at 25 °C), and insulin from bovine pancreas in powder form (CAS 11070-73-8) were purchased from Sigma-Aldrich (St. Louis, MO). HEPES free acid, potassium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and sodium cacodylate in powder form were also purchased from Sigma-Aldrich. The high-performance liquid chromatography-purified DNA oligonucleotides were custom-synthesized by The Midland Certified Reagent Co. (Midland, TX). The DNA sequence used was 5′-$(ACAG_4TGTG_4)_2$-3′.

Preparation of Samples. For use in ITC and DSC measurements, the oligonucleotides were dissolved in a buffer consisting of 10 mM phosphate and 100 mM KCl (PBS). Some ITC experiments were also performed in buffers that consisted of 10 mM HEPES and 100 mM KCl or 10 or 100 mM Tris-HCl and 100 mM KCl. Additional experiments were conducted with buffers that consisted of 10 mM sodium cacodylate and 100 mM KCl or 20 mM PIPES and 100 mM KCl. In each case, the buffer pH was 7.4. All buffer solutions were made using ultrapure water (18.2 MΩ cm^{-1} resistivity) obtained from a Barnstead water purifier (Fisher Scientific) and were filtered (0.2 μm pore size) prior to being used. DNA oligonucleotide samples prepared in buffer were heated to 90 °C and held at this temperature for 10 min, after which they were cooled at a rate of 0.5 °C/min to 5 °C using a thermocycler (MJ Research PTC-150).

Circular Dichroism (CD) Spectroscopy. The conformations of the DNA structures studied by DSC and ITC were examined using an Olis (Bogart, GA) DSM 20 spectrophotometer. All tests were performed at room temperature in a spectral range of 200−350 nm with 1 mm quartz cuvettes. PBS was set as the baseline.

Differential Scanning Calorimetry. Differential scanning calorimetry measurements were performed using a Nano DSC instrument from TA Instruments (Lindon, UT). Experiments were performed in PBS. Buffer was degassed for 25 min at room temperature prior to being used. The sample and reference cells were loaded with the buffer solution and equilibrated for ∼30 min until the baseline power difference remained within a tenth of a microwatt over 5 min before a buffer versus buffer scan was initiated. Scans were repeated at a rate of 0.5 °C/min in the range of 25−110 °C to condition the cells. A typical experiment consisted of first obtaining a buffer versus buffer scan and then replacing the sample cell with a solution of insulin and/or DNA and equilibrating the cells for 30 min prior to another scan. Each sample scan was prepared for analysis by subtracting the buffer versus buffer scan and converting to molar heat capacity data. All data were recorded with the TA Instruments software, and further analysis was performed using TA NanoAnalyze version 2.1.13. For all experiments, the insulin concentration was 324 μM, and the DNA concentration was in the range of 70−248 μM. Analysis was performed using a two-state scaled model. Statistical analysis was performed on the transition temperature(s) with a 95% confidence interval.

Isothermal Titration Calorimetry. All experiments were performed on a Nano ITC Standard Volume instrument from TA Instruments. Binding conditions were optimized for the insulin−G-quadruplex system. Experiments were performed primarily in PBS. Other experiments used buffers that consisted of 10 mM HEPES, 10 mM sodium cacodylate, and 20 mM PIPES or 10 or 100 mM Tris-HCl all containing 100 mM KCl (pH 7.4). Buffers were degassed for 25 min at room temperature prior to experiments. The reference cell in the ITC was filled with degassed ultrapure water (18.2 MΩ cm^{-1} resistivity). In a multiple-injection mode, the experiment was set up at temperatures of 10, 20, 25, 30, and 37 °C for 20 injections (12 μL each) with 700 or 1000 s injection intervals. The stir rate was 250 rpm. Once the cell had been filled and the syringe loaded, stirring was turned on and the ITC apparatus was allowed to autoequilibrate. Control experiments were performed with the injection of G-quadruplex DNA into PBS buffer at pH 7.4 and with PBS buffer into insulin. All data were recorded with the TA Instruments software provided, ITCRun version 2.2.3. Further analysis was performed using TA NanoAnalyze version 2.4.1. The area under each peak was

dx.doi.org/10.1021/jp411293r | J. Phys. Chem. B 2014, 118, 1784−1790

integrated, and the resulting data were modeled using an independent model fit with variables ΔH, n, and K_A. The change in free energy and the change in entropy were determined by the relationship $\Delta G = -RT \ln K_A = \Delta H - T\Delta S$. Statistical analysis was then performed on the thermodynamic parameters with a 95% confidence interval.

RESULTS AND DISCUSSION

The thermodynamics of the binding interaction between insulin and the G-quadruplex formed by the ILPR consensus sequence were characterized in this study. To confirm G-quadruplex formation, CD spectroscopy was performed. G-quadruplexes with an antiparallel conformation typically display a positive signal at ~295 nm and a negative signal at ~260 nm; quadruplexes with a parallel conformation generally show a positive signal at ~265 nm and a negative signal at ~240 nm. A positive peak found at around 295 nm and a shoulder at 260 nm are thought to indicate a mixed parallel and antiparallel conformation.[6,12] The CD spectrum (Figure 1, inset) for the consensus ILPR sequence confirms the formation of a mixed parallel and antiparallel quadruplex.[12b] The DSC thermogram of this sequence shown in Figure 1 indicates a biphasic melting curve with melting transitions at 71.9 and 93.2 °C. The biphasic melting of the ILPR consensus sequence has also been confirmed by CD melting studies using a 31-mer ILPR sequence, $(ACAG_4TGTG_4)_2ACA$, showing transition temperatures at ~78 and ~88 °C.[3a] The lower T_m found in these studies is attributed to the melting of the parallel conformation, while the higher T_m is attributed to the melting of the antiparallel conformation.[3a] In UV melting studies of the ILPR consensus sequence, a T_m of 67.1 °C was reported.[13] Under the conditions used in the latter study, the DNA sequence showed predominantly an antiparallel conformation based on the CD spectrum.[13] The absence of the parallel conformation in the CD spectrum might be due to the low salt concentration used (i.e., 2.5 mM KCl). Nonetheless, a UV melting T_m of 92 °C (data not shown) was also reported for the ILPR consensus sequence in which both parallel and antiparallel conformations were confirmed by CD measurements.[6] Taken together, these results suggest that the population of the parallel quadruplex conformation in the ILPR consensus sequence may be highly sensitive to conditions such as buffer and salt concentrations. It is likely, therefore, that the different populations of the parallel/antiparallel G-quadruplex conformations may have accounted for the differences observed in the CD melting and UV melting temperatures in these studies. On the basis of our DSC results and prior studies that demonstrated a greater thermodynamic stability for the antiparallel conformation,[3a] the T_m observed here at 71.9 °C is attributed to the parallel conformation and the T_m at 93.2 °C to the antiparallel conformation.

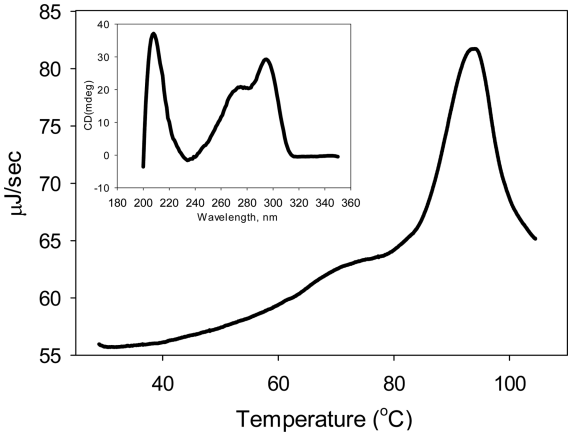


Figure 1. DSC thermogram of the ILPR consensus DNA sequence. The DNA concentration was 70 μM in PBS (pH 7.4). The scan rate was 0.5 °C/min. First- and second-derivative plots are shown in Figure S1 of the Supporting Information. The inset shows circular dichroism spectrum of the ILPR consensus DNA sequence. The DNA concentration was 10 μM in PBS (pH 7.4) at 25 °C.

Following confirmation of G-quadruplex formation, DSC thermograms were acquired for two separate systems: insulin alone and insulin bound to the ILPR consensus sequence (Figure 2).

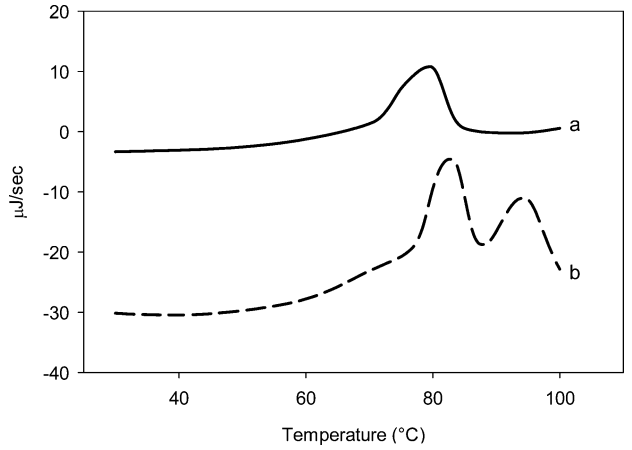


Figure 2. DSC thermograms of (a) insulin and (b) the insulin–DNA complex. The solid line represents insulin with an unfolding event displaying a transition maximum at 78.7 °C. The dashed line represents insulin and the ILPR consensus DNA sequence, where insulin unfolds at 82.5 °C and DNA unfolds at 93.9 °C. Concentrations of 324 μM insulin and 248 μM DNA in PBS (pH 7.4) were used. The scan rate was 0.5 °C/min. First- and second-derivative plots are shown in Figure S2 of the Supporting Information.

Data were analyzed using a two-state scaled model, which resulted in a transition temperature of 78.68 °C for insulin alone (see Table 1). Upon introduction of the ILPR DNA, the denaturation temperature of insulin was increased to 82.46 °C (Table 1). An increase of nearly 4 °C in insulin denaturation temperature upon association with the G-quadruplex DNA of the ILPR consensus sequence implies a significant stabilization of insulin and suggests that binding has occurred.[14]

Table 1. Phase Transition Temperatures Determined by DSC for Insulin, DNA, and the Insulin–DNA Complex

	insulin T_m	DNA T_m
insulin alone	78.68 ± 0.02	–
insulin and DNA	82.46 ± 0.02	93.90 ± 0.05
DNA alone	–	93.20 ± 0.03

Following the use of DSC to confirm the binding of insulin to G-quadruplex DNA, ITC measurements were performed to determine the thermodynamic parameters, including the equilibrium binding constant (K_D) and changes in enthalpy (ΔH), Gibbs free energy (ΔG), and entropy (ΔS). The ITC raw data for the binding of insulin to the ILPR consensus sequence at the representative temperature of 25 °C are shown

dx.doi.org/10.1021/jp411293r | J. Phys. Chem. B 2014, 118, 1784–1790

in Figure 3a. The ITC data confirm the observation from DSC and previous studies that the G-quadruplex formed by the ILPR

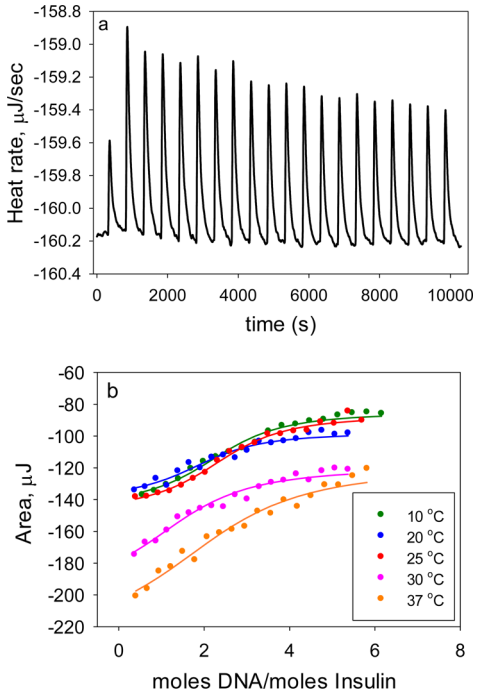


Figure 3. Isothermal titration calorimetry analysis of binding of insulin to the ILPR consensus DNA sequence. (a) Raw ITC data at 25 °C. (b) Plots of integrated calorimetric data after control subtraction at 10, 20, 25, 30, and 37 °C fit with an independent binding model. DNA (572−647 μM) in 12 μL aliquots was titrated into the cell containing 30 μM insulin in PBS (pH 7.4). A first small injection (6 μL) was disregarded when data were analyzed.

consensus sequence binds to insulin. Exothermic binding association was observed at all temperatures investigated in this study. The integrated ITC binding curves for the interaction between insulin and the ILPR consensus sequence from 10 to 37 °C are shown in Figure 3b. The binding isotherms were fit to an independent model (single-site binding model) to determine the binding stoichiometry (n), ΔH, and K_A. Furthermore, these values allowed the calculation of ΔG and ΔS. The thermodynamic parameters for this interaction are listed in Table 2. Recently, a fluorescence quenching study of the ILPR sequence with insulin assumed a 1:1 binding stoichiometry based on the linearity of the double-reciprocal plot.[6] A similar 1:1 binding model was also assumed by Xiao and co-workers in an SPR study of the binding of insulin to ILPR G-quadruplex DNA.[10] Although these previous studies have assumed 1:1 binding between insulin and ILPR G-quadruplex DNA, the ITC data shown in Figure 3b were fit with a variable n model as well as with n = 1 (see Figure S3 of the Supporting Information). The ITC data did not appear to fit well with the n = 1 independent binding model for all data obtained from 10 to 37 °C. Varying n as a fitting parameter results in much better fits and yields a binding stoichiometry of ∼2, suggesting that a G-quadruplex is more likely to bind to insulin dimers rather than insulin monomers. This result is consistent with the conditions used in this study, under which insulin is known to exist in dimeric form.[15] Moreover, as insulin is known to exist mainly in the hexameric form *in vivo*, more complex interactions are certainly feasible for this system. Nevertheless, the results of ITC data analysis assuming a 1:1 binding stoichiometry are reported in Table S1 of the Supporting Information for comparison.

On the basis of the data shown in Table 2 and Figure 4a, the enthalpy of the binding interaction decreased from −6 to −13

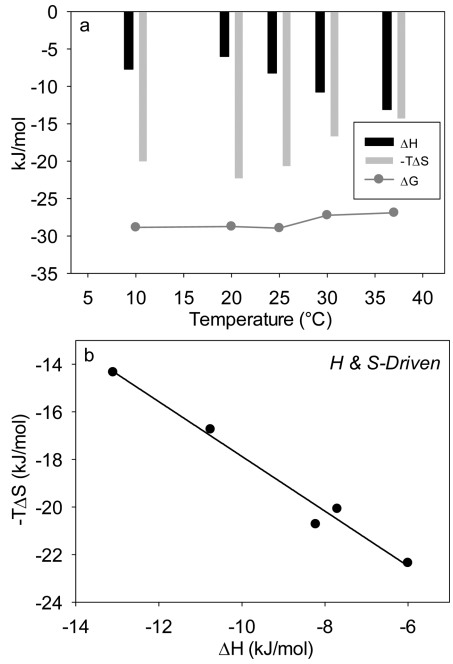


Figure 4. (a) Thermodynamic parameters obtained from ITC data, including $-T\Delta S$, ΔH, and ΔG, for the interaction between insulin and the ILPR consensus DNA sequence at 10, 20, 25, 30, and 37 °C. (b) Enthalpy−entropy compensation plot for the binding of insulin to the ILPR consensus DNA sequence at all temperatures studied (10−37 °C). The linear regression is given by $-T\Delta S$ (kJ/mol) = (−1.0 ± 0.1)ΔH (kJ/mol) − 28.5 ± 1.1 (kJ/mol).

kJ/mol as the temperature was increased from 10 to 37 °C, while the entropic term increased from −22 to −14 kJ/mol with an increase in temperature. This suggests that the binding of insulin to the ILPR consensus sequence is mainly entropically driven at low temperatures (<30 °C) but becomes roughly equally driven by both entropic and enthalpic terms at

Table 2. Thermodynamic Parameters for the Binding of ILPR G-Quadruplex DNA (consensus sequence) and Insulin in PBS (pH 7.4)

T (°C)	n	K_D (μM)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	$-T\Delta S$ (kJ mol^{-1})
10	2.43 ± 0.17	8.8 ± 1.4	−7.7 ± 0.8	−27.4 ± 0.4	−19.7 ± 0.9
20	2.08 ± 0.32	9.2 ± 1.4	−6.0 ± 1.5	−28.2 ± 0.4	−22.3 ± 1.5
25	2.51 ± 0.10	8.4 ± 0.9	−8.2 ± 0.5	−28.9 ± 0.3	−20.7 ± 0.6
30	1.82 ± 0.37	16.9 ± 2.3	−10.8 ± 3.8	−27.7 ± 0.3	−16.9 ± 3.8
37	2.33 ± 0.31	19.4 ± 3.0	−13.1 ± 2.6	−28.0 ± 0.4	−14.9 ± 2.6

dx.doi.org/10.1021/jp411293r | J. Phys. Chem. B 2014, 118, 1784−1790

higher temperatures (~37 °C). These two competing effects compensate for the Gibbs free energy, which appears to change very little over the temperature range of 10−37 °C (Figure 4).

For the results presented here, the dissociation constant, K_D, ranges from 8.4 to 19.4 μM, representing a >2-fold change over the temperature range examined. Moreover, the K_D values obtained from the ITC measurements are 1 order of magnitude higher than those obtained from measurements by SPR (K_D ~ 0.2−0.7 μM)[10] and fluorescence quenching (0.17 μM).[6] Such variances could be due to the differences in the buffers used (see below) in the measurements and/or how binding measurements are performed by these methods. The differences in K_D values may also suggest a protonation−deprotonation effect in the binding pocket. To consider the feasibility of the latter possibility, the ILPR consensus sequence−insulin association was examined by ITC in six different buffers at 25 °C. The buffers phosphate, HEPES, and Tris-HCl at a low concentration (10 mM) showed binding affinity, while the buffers PIPES, sodium cacodylate, and Tris-HCl at a high concentration (100 mM) showed no binding affinity. Furthermore, the 10 mM Tris-HCl buffer displayed inconsistent control ITC runs, suggesting that this buffer may be very sensitive to slight differences in pH between the ligand and sample. Using the enthalpy of ionization (ΔH_{ion}) of the phosphate and HEPES buffers and the observed enthalpy of heat from the binding interaction (ΔH_{obs}), the number of protons released upon binding (n_{H^+}) was determined. ΔH_{obs} and ΔH_{ion} are related by the equation[16] $\Delta H_{obs} = \Delta H_{bind} + (n_{H^+})\Delta H_{ion}$, where ΔH_{bind} is the enthalpy of the intrinsic binding between insulin and ILPR G-quadruplex DNA. On the basis of this relationship, approximately three protons are released to the buffer in the binding process and the intrinsic binding enthalpy is estimated to be −2.50 kJ/mol (see Figure 5). The accuracy of these values can be improved by using

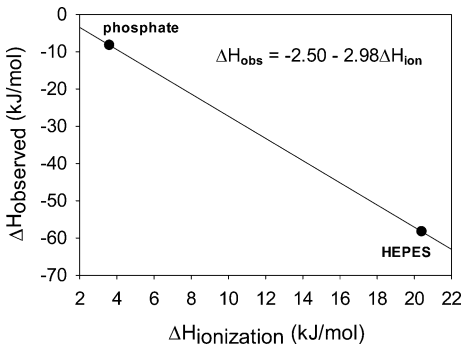


Figure 5. Plot of ΔH_{obs} versus ΔH_{ion} for the insulin−G-quadruplex DNA binding interaction performed in phosphate and HEPES buffer systems at 25 °C and pH 7.4. The negative slope of the line (n_{H^+}) provides the number of protons released to the buffer upon binding, which is equal to 3. The intercept is −2.50 kJ/mol, which represents the intrinsic binding enthalpy (ΔH_{bind}).

more data points in Figure 5. The inclusion of ΔH_{obs} in 10 mM Tris-HCl buffer shows the number of protons released was ~4 as shown in Figure S4 of the Supporting Information. However, given the uncertainty associated with ΔH_{obs} obtained in the Tris-HCl buffer at a low concentration, this data point was omitted in the analysis performed in Figure 5. The influence of the protonation−deprotonation effect on ΔH_{obs} was also examined by determining the van't Hoff enthalpy (ΔH_{vH}) from a plot of ln K_D versus inverse temperature in Figure S5 of the Supporting Information. The slope of the plot was multiplied by the gas constant R to yield a value for ΔH_{vH} of −23.0 ± 9.7 kJ/mol. The van't Hoff enthalpy is significantly more negative than the calorimetric enthalpy (ΔH_{obs}). Such large differences between ΔH_{vH} and ΔH_{obs} have been observed previously.[17] Although we cannot rule out the possibility that protonation−deprotonation effects contribute to ΔH_{obs}, the differences between ΔH_{vH} and ΔH_{obs} have also been explained in terms of statistical errors in the measurements,[18] flaws in the background correction in ITC,[19] and desolvation effects.[20]

The magnitude of the change in heat capacity (ΔC_p) is generally thought to be related to the nature of the hydrophobic interactions involved in binding processes. Specific interactions that occur upon association of the protein with DNA have been shown to demonstrate large negative changes in standard heat capacity.[21] For the insulin−ILPR DNA interaction, the ΔC_p was found to be −422.7 J K^{-1} mol^{-1} for the temperature range of 20−37 °C (Figure 6). The change

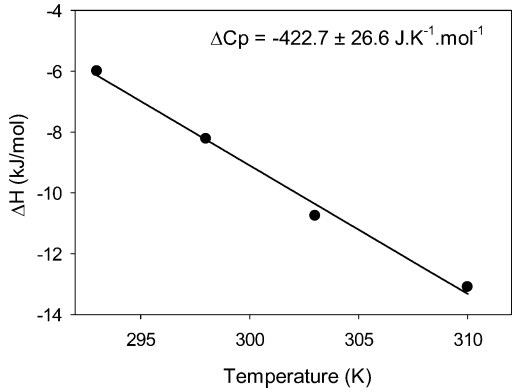


Figure 6. Heat capacity plot for the binding of insulin to the ILPR DNA consensus sequence at 20, 25, 30, and 37 °C. Using linear regression, the change in heat capacity (ΔC_p) for this interaction is equal to −422.7 ± 26.6 J K^{-1} mol^{-1}.

in heat capacity measured in protein−ligand binding has been attributed to hydrophobic and vibrational effects.[22] Hydrophobic effects are thought to arise from the water cage surrounding nonpolar functional groups in the binding region, while vibrational effects are likely to be caused by changes in the number of internal vibrational modes of the protein structure.[23] Previous studies have indicated that a negative ΔC_p for protein−ligand interactions dominated by entropy at lower temperatures but by enthalpy at higher temperatures is a strong indication of the involvement of hydrophobic interactions in the binding region.[22] To determine the extent to which hydrophobic and vibrational effects contribute to ΔC_p and ΔS in this system, the hydrophobic and vibrational components of ΔC_p and ΔS were determined on the basis of the method described by Sturtevant[23] and Gilli et al.[22] and are listed in Table 3. The results from Table 3 indicate that hydrophobic effects are dominant compared to vibrational effects in the interaction of insulin with ILPR DNA. Specifically, the G-quadruplex binding interaction takes place with the β subunit of the insulin protein at amino acid sequence VCG(N)RGF, as shown in previous work by Xiao and McGown.[24] More recent studies have also shown that G-quadruplex structures have preferential binding to the β structures of proteins because of the highly negative charge density of G-quadruplex DNA.[25] The dominant hydrophobic effects listed in Table 3 are

dx.doi.org/10.1021/jp411293r | J. Phys. Chem. B 2014, 118, 1784−1790

Table 3. Changes in Unitary Entropy (ΔS_u) and Heat Capacity (ΔC_p) Resulting from Hydrophobic (hydro) and Vibrational (vib) Effects for the Binding of Insulin and the ILPR G-Quadruplex (consensus sequence)

T (°C)	ΔC_p(hydro) (J K^{-1} mol^{-1})	ΔC_p(vib) (J K^{-1} mol^{-1})	ΔS_u(hydro) (J K^{-1} mol^{-1})	ΔS_u(vib) (J K^{-1} mol^{-1})
20	−423	−0.5	110	−0.5
25	−418	−5.4	109	−5.6
30	−407	−15.8	106	−16.6
37	−401	−21.8	104	−22.9

consistent with this finding as the valine, cysteine, glycine, and phenylalanine residues are hydrophobic under experimental conditions. As asparagine and arginine are more hydrophilic, it is possible that the structure of this sequence allows amino acids at its ends to interact more closely with the ILPR G-quadruplex DNA during the binding process. In addition to this hydrophobic effect, protonation and deprotonation also contribute to large negative changes in heat capacity.[26] The explicit contribution of this effect to heat capacity is beyond the scope of this study and requires changes in solution conditions, which will be explored in future work. Nonetheless, the large negative change in heat capacity obtained for the interaction between ILPR DNA and insulin is likely to contain contributions from both hydrophobic and protonation−deprotonation effects.

In summary, we examined the binding of insulin to the G-quadruplex DNA formed from the consensus DNA sequence found in the insulin-linked polymorphic region. The G-quadruplex structures were established by CD spectroscopy, and binding studies were performed by DSC and ITC. K_D values measured show a moderate binding affinity over the range of 10−37 °C. The binding interaction was dominated by entropic factors at low temperatures and enthalpic factors at higher temperatures, displaying an enthalpy−entropy compensation across the range of temperatures studied. Protonation effects were examined in two buffers with different ionization enthalpies under identical experimental conditions, which suggested the release of three protons upon binding of G-quadruplex DNA to insulin with an intrinsic binding enthalpy of −2.5 kJ/mol. Finally, the change in heat capacity suggested a hydrophobic dominance in the binding region. This can be attributed to the amino acid sequence of insulin's β subunit, which contains several strongly hydrophobic amino acid groups. Taken together, these results provide further insights into the physical mechanisms that control the association between insulin and the G-quadruplexes formed by ILPR DNA.

■ ASSOCIATED CONTENT

Supporting Information

Differentiation of the DSC thermogram shown in Figure 1 (Figure S1), differentiation of DSC thermograms shown in Figure 2 (Figure S2), isothermal titration calorimetry analysis of the binding of insulin to the ILPR consensus DNA sequence (Figure S3), plot of ΔH_{obs} versus ΔH_{ion} for the insulin−G-quadruplex DNA sequence binding interaction performed in phosphate, HEPES, and 10 mM Tris-HCl buffer systems at 25 °C and pH 7.4 (Figure S4), plot of ln K_D versus inverse temperature to determine the van't Hoff enthalpy (ΔH_{vH}) based on ITC data for the interaction between insulin and the ILPR consensus DNA sequence (Figure S5), and thermodynamic parameters for the binding of ILPR G-quadruplex DNA (consensus sequence) and insulin in PBS (pH 7.4) with n (binding stoichiometry) = 1 (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ksinniah@calvin.edu. Phone: (616) 526-6058. Fax: (616) 526-6501.

Author Contributions

C.M.T. and N.L.M. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

K.S. acknowledges the support of the National Science Foundation (MCB-1022022) and the Howard Hughes Medical Institute (student support) at Calvin College. We thank Professor Moses Lee (Hope College, Holland, MI) for kind use of the CD spectrometer.

■ REFERENCES

(1) Kumar, N.; Maiti, S. A Thermodynamic Overview of Naturally Occurring Intramolecular DNA Quadruplexes. *Nucleic Acids Res.* **2008**, *36* (17), 5610−5622.

(2) (a) Fry, M. Tetraplex DNA and Its Interacting Proteins. *Front. Biosci., Landmark Ed.* **2007**, *12*, 4336−4351. (b) Maizels, N.; Gray, L. T. The G4 Genome. *PLoS Genet.* **2013**, *9* (4), e1003468.

(3) (a) Yu, Z.; Schonhoft, J. D.; Dhakal, S.; Bajracharya, R.; Hegde, R.; Basu, S.; Mao, H. Ilpr G-Quadruplexes Formed in Seconds Demonstrate High Mechanical Stabilities. *J. Am. Chem. Soc.* **2009**, *131* (5), 1876−1882. (b) Lynch, S.; Baker, H.; Byker, S. G.; Zhou, D.; Sinniah, K. Single Molecule Force Spectroscopy on G-Quadruplex DNA. *Chem. Eur. J.* **2009**, *15* (33), 8113−8116.

(4) Biffi, G.; Tannahill, D.; McCafferty, J.; Balasubramanian, S. Quantitative Visualization of DNA G-Quadruplex Structures in Human Cells. *Nat. Chem.* **2013**, *5* (3), 182−186.

(5) Pagano, B.; Mattia, C. A.; Giancola, C. Applications of Isothermal Titration Calorimetry in Biophysical Studies of G-Quadruplexes. *Int. J. Mol. Sci.* **2009**, *10* (7), 2935−2957.

(6) Schonhoft, J. D.; Das, A.; Achamyeleh, F.; Samdani, S.; Sewell, A.; Mao, H.; Basu, S. Ilpr Repeats Adopt Diverse G-Quadruplex Conformations That Determine Insulin Binding. *Biopolymers* **2010**, *93* (1), 21−31.

(7) (a) Connor, A. C.; Frederick, K. A.; Morgan, E. J.; McGown, L. B. Insulin Capture by an Insulin-Linked Polymorphic Region G-Quadruplex DNA Oligonucleotide. *J. Am. Chem. Soc.* **2006**, *128* (15), 4986−4991. (b) Hammond-Kosack, M. C.; Dobrinski, B.; Lurz, R.; Docherty, K.; Kilpatrick, M. W. The Human Insulin Gene Linked Polymorphic Region Exhibits an Altered DNA Structure. *Nucleic Acids Res.* **1992**, *20* (2), 231−236.

(8) Kennedy, G. C.; German, M. S.; Rutter, W. J. The Minisatellite in the Diabetes Susceptibility Locus Iddm2 Regulates Insulin Transcription. *Nat. Genet.* **1995**, *9* (3), 293−298.

(9) Melloul, D.; Marshak, S.; Cerasi, E. Regulation of Insulin Gene Transcription. *Diabetologia* **2002**, *45* (3), 309−326.

(10) Xiao, J.; Carter, J. A.; Frederick, K. A.; McGown, L. B. A Genome-Inspired DNA Ligand for the Affinity Capture of Insulin and Insulin-Like Growth Factor-2. *J. Sep. Sci.* **2009**, *32* (10), 1654−1664.

(11) Gerasimov, J. Y.; Schaefer, C. S.; Yang, W.; Grout, R. L.; Lai, R. Y. Development of an Electrochemical Insulin Sensor Based on the Insulin-Linked Polymorphic Region. *Biosens. Bioelectron.* **2013**, *42*, 62−68.

(12) (a) Paritala, H.; Firestine, S. M. Characterization of Insulin Ilpr Sequences for Their Ability to Adopt a G-Quadruplex Structure. *Nucleosides, Nucleotides Nucleic Acids* **2010**, *29* (2), 81−90. (b) Schonhoft, J. D.; Bajracharya, R.; Dhakal, S.; Yu, Z.; Mao, H.;

Basu, S. Direct Experimental Evidence for Quadruplex-Quadruplex Interaction within the Human Ilpr. *Nucleic Acids Res.* **2009**, *37* (10), 3310−3320.
(13) Bauer, L.; Tluckova, K.; Tohova, P.; Viglasky, V. G-Quadruplex Motifs Arranged in Tandem Occurring in Telomeric Repeats and the Insulin-Linked Polymorphic Region. *Biochemistry* **2011**, *50* (35), 7484−7492.
(14) Jelesarov, I.; Bosshard, H. R. Isothermal Titration Calorimetry and Differential Scanning Calorimetry as Complementary Tools to Investigate the Energetics of Biomolecular Recognition. *J. Mol. Recognit.* **1999**, *12* (1), 3−18.
(15) Huus, K.; Havelund, S.; Olsen, H. B.; van de Weert, M.; Frokjaer, S. Thermal Dissociation and Unfolding of Insulin. *Biochemistry* **2005**, *44* (33), 11171−11177.
(16) Leavitt, S.; Freire, E. Direct Measurement of Protein Binding Energetics by Isothermal Titration Calorimetry. *Curr. Opin. Struct. Biol.* **2001**, *11* (5), 560−566.
(17) (a) Liu, Y.; Sturtevant, J. M. Significant Discrepancies between Van't Hoff and Calorimetric Enthalpies. II. *Protein Sci.* **1995**, *4* (12), 2559−2561. (b) Naghibi, H.; Tamura, A.; Sturtevant, J. M. Significant Discrepancies between Van't Hoff and Calorimetric Enthalpies. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (12), 5597−5599. (c) Liu, Y.; Sturtevant, J. M. Significant Discrepancies between Van't Hoff and Calorimetric Enthalpies. III. *Biophys. Chem.* **1997**, *64* (1−3), 121−126.
(18) Chaires, J. B. Possible Origin of Differences between Van't Hoff and Calorimetric Enthalpy Estimates. *Biophys. Chem.* **1997**, *64* (1−3), 15−23.
(19) Mizoue, L. S.; Tellinghuisen, J. Calorimetric vs. Van't Hoff Binding Enthalpies from Isothermal Titration Calorimetry: Ba^{2+}-Crown Ether Complexation. *Biophys. Chem.* **2004**, *110* (1−2), 15−24.
(20) Castellano, B. M.; Eggers, D. K. Experimental Support for a Desolvation Energy Term in Governing Equations for Binding Equilibria. *J. Phys. Chem. B* **2013**, *117* (27), 8180−8188.
(21) (a) Ha, J. H.; Spolar, R. S.; Record, M. T., Jr. Role of the Hydrophobic Effect in Stability of Site-Specific Protein-DNA Complexes. *J. Mol. Biol.* **1989**, *209* (4), 801−816. (b) Chen, S. H.; Suzuki, C. K.; Wu, S. H. Thermodynamic Characterization of Specific Interactions between the Human Lon Protease and G-Quartet DNA. *Nucleic Acids Res.* **2008**, *36* (4), 1273−1287.
(22) Gilli, P.; Gilli, G.; Borea, P. A.; Varani, K.; Scatturin, A.; Dalpiaz, A. Binding Thermodynamics as a Tool to Investigate the Mechanisms of Drug-Receptor Interactions: Thermodynamics of Cytoplasmic Steroid/Nuclear Receptors in Comparison with Membrane Receptors. *J. Med. Chem.* **2005**, *48* (6), 2026−2035.
(23) Sturtevant, J. M. Heat Capacity and Entropy Changes in Processes Involving Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74* (6), 2236−2240.
(24) Xiao, J.; McGown, L. B. Mass Spectrometric Determination of Ilpr G-Quadruplex Binding Sites in Insulin and Igf-2. *J. Am. Soc. Mass Spectrom.* **2009**, *20* (11), 1974−1982.
(25) (a) Tsukakoshi, K.; Abe, K.; Sode, K.; Ikebukuro, K. Selection of DNA Aptamers That Recognize α-Synuclein Oligomers Using a Competitive Screening Method. *Anal. Chem.* **2012**, *84* (13), 5542−5547. (b) Yoshida, W.; Saito, T.; Yokoyama, T.; Ferri, S.; Ikebukuro, K. Aptamer Selection Based on G4-Forming Promoter Region. *PLoS One* **2013**, *8* (6), e65497.
(26) Kozlov, A. G.; Lohman, T. M. Large Contributions of Coupled Protonation Equilibria to the Observed Enthalpy and Heat Capacity Changes for ssDNA Binding to *Escherichia coli* SSB Protein. *Proteins* **2000**, No. Suppl. 4, 8−22.