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Thermodynamic Characterization of Binding *Oxytricha nova* Single Strand Telomere DNA with the Alpha Protein N-terminal Domain

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Abstract

The *Oxytricha nova* telomere binding protein alpha subunit binds single strand DNA and participates in a nucleoprotein complex that protects the very ends of chromosomes. To understand how the N-terminal, DNA binding domain of alpha interacts with DNA we measured the stoichiometry, enthalpy (ΔH), entropy (ΔS), and dissociation constant (K_{D-DNA}) for binding telomere DNA fragments at different temperatures and salt concentrations using native gel electrophoresis and isothermal titration calorimetry (ITC). About 85% of the total free energy of binding corresponded with non-electrostatic interactions for all DNAs. Telomere DNA fragments d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), and d(G₄T₄G₄) each formed monovalent protein complexes. In the case of d(T₄G₄T₄G₄), which has two tandemly repeated d(TTTTGGGG) telomere motifs, two binding sites were observed. The high-affinity “A site” has a dissociation constant, $K_{D-DNA(A)}=13(\pm 4)$ nM, while the low-affinity “B site” is characterized by $K_{D-DNA(B)}=5600(\pm 600)$ nM at 25 °C. Nucleotide substitution variants verified that the A site corresponds principally with the 3'-terminal portion of d(T₄G₄T₄G₄). The relative contributions of entropy (ΔS) and enthalpy (ΔH) for binding reactions were DNA length-dependent as was heat capacity (ΔC_p). These trends with respect to DNA length likely reflect structural transitions in the DNA molecule that are coupled with DNA–protein association. Results presented here are important for understanding early intermediates and subsequent stages in the assembly of the full telomere nucleoprotein complex and how binding events can prepare the telomere DNA for extension by telomerase, a critical event in telomere biology.

Keywords

telomere-binding protein; telomere biology; protein–single strand DNA interactions; ITC; binding energetics

Introduction

Telomere DNA consists of tandem repeats of a simple T and G-rich sequence such as d(TTGGGG) in *Tetrahymena thermophila*, d(TTAGGG) in vertebrates and some fungi, and d(TTTTGGGG) in *Oxytricha nova* and *Euplotes crassus*.^{1–4} The 3'-terminal strand extends past the duplex DNA creating a single strand DNA extension that can form lariat DNA structures, seen by electron microscopy,^{5–9} through interaction with internal double-stranded repeats and

proteins in humans, chickens, and developing macronuclei of ciliates. In mature macronuclei of ciliates, where the length of both duplex and single strand telomere DNA is precise and short, single strand DNA binds with telomere end-specific proteins to form a protective nucleoprotein complex.¹⁰⁻¹⁴ Sequence and structural homology among telomere end binding proteins¹⁵ and direct tests using gene deletion studies in yeast¹⁶ or RNA-interference in *Stylonychia lemnae*¹⁷ indicate that telomere end binding proteins are essential for telomere function and cell viability, sequestering telomere DNA and coordinating timely exchange with telomerase.

The simple, well-defined telomeres of ciliates provide a unique opportunity for understanding telomere complex assembly and disassembly. *Oxytricha nova* single strand telomere DNA consists of two d(TTTTGGGG) repeats and associates with alpha and beta subunits of the telomere end binding protein, OnTEBP.¹²⁻¹⁴ Short telomere DNA fragments form binary complexes with the alpha N-terminal DNA-binding domain. Since the alpha N-terminal domain retains DNA binding properties measured for alpha in the absence of beta,^{13,18} these DNA complexes likely represent early intermediates in a step-wise assembly pathway.^{19,20} A co-crystal structure of telomere-derived DNA complexed with the alpha N-terminal domain provided atomic details for DNA-protein interactions showing how this DNA-binding domain establishes contacts consistent with 3'-terminal and sequence-specific recognition of DNA.^{19,20}

In order to understand early steps in assembly of DNA-protein telomere end structures, we have measured the stoichiometry (n), enthalpy (ΔH), entropy (ΔS), and dissociation constant (K_{D-DNA}) for binding telomere DNA fragments with the alpha protein N-terminal domain at different temperatures and salt concentrations using native gel electrophoresis and isothermal titration calorimetry (ITC). These measures complement and extend what we know about DNA-protein interactions for a sequence-specific single strand DNA binding protein and for T and G-rich DNA substrates. Short d(T₂G₄) and d(T₄G₄) DNAs bound the alpha N-terminal domain with a modest negative heat capacity change of binding. Longer DNAs showed systematic trends with respect to enthalpy and entropy of protein binding. As G nucleotides were added to the 5' end of the d(T₄G₄) DNA, binding was associated with increasingly favorable positive entropy contributions compensated by decreases in the negative enthalpy contribution, consistent with the idea that these longer telomere-derived DNAs form hairpin structures stabilized by G•G base-pairs in their uncomplexed state.²¹ Binding with the alpha N-terminal domain resolves these structures, and in the case of d(T₄G₄T₄G₄) DNA, exposes a second protein-binding site. Binding at 3'-terminal and 3'-distal sites may form the basis of higher-order structures and may play an important role in ensuring that DNA remains in an extended conformation suitable for primer utilization by telomerase.

Results

Stoichiometry of DNA binding

To determine the stoichiometry of protein binding with DNA containing single or double d(T₄G₄) telomere repeats we examined the electrophoretic mobility of the alpha N-terminal domain as a function of DNA concentration for d(T₄G₄), d(G₃T₄G₄) and d(T₄G₄T₄G₄) DNAs. Figure 1 shows Coomassie-stained non-denaturing acrylamide gels with bands indicating the mobility of free and DNA-bound protein. Increasing the concentration of d(T₄G₄) and d(G₃T₄G₄) shifted the free protein (band *a*) to a new position corresponding to a monovalent DNA complex (band *b*). The d(T₄G₄T₄G₄) DNA produced two shifted bands. One of these d(T₄G₄T₄G₄)-shifted bands corresponds to a negatively charged DNA complex that continually increased in intensity with respect to DNA concentration (band *b*, in Figure 1(c)). The second d(T₄G₄T₄G₄)-shifted band corresponds to a positively charged DNA complex that is only observed at DNA:protein ratios less than 1:1 (band *c*, in Figure 1(c)).

Assignment of the two d(T₄G₄T₄G₄)-shifted bands follows from consideration of size and charge differences characterizing the uncomplexed protein and DNA–protein complexes. We assign band *c* in Figure 1(c) to a protein•DNA•protein complex since band *c* is positioned midway between that of the free protein (band *a*) and the species encountered with excess DNA (band *b*). Note that band *c* is only observed when DNA is limiting. As more DNA is added, protein in the protein•DNA•protein complex repartitions to form a DNA•protein complex (band *b*). Similar results were observed for the full-length alpha subunit (Figure 1(d)–(f)), indicating that the presence or absence of the alpha C-terminal domain does not change the stoichiometry of binding DNA and that the full-length alpha subunit also forms a protein•DNA•protein complex when DNA binding sites are limiting. These results are consistent with previous characterization of DNA–alpha complexes by gel electrophoresis that showed two alpha proteins can bind one d(T₄G₄T₄G₄) DNA molecule.²²

Stoichiometry of DNA binding was further evaluated by ITC. Titration of protein into solutions of DNA resulted in well-resolved binding phases and the heat of final injections corresponded with that observed in control titrations of protein into buffer. The reverse titration, injection of DNA into a solution of protein, however, resulted in thermograms for which the heats of final injections were exothermic while the heats of control titrations of DNA into buffer were endothermic. The non-correspondence of final injections can be attributed to a combination of dissociating non-specific DNA–DNA interactions upon dilution and non-specific aggregation upon mixing concentrated DNA solutions with protein. This aggregation effect is visible as a cloudy precipitate that quickly forms and then dissolves as routinely observed in preparation of diffraction quality single crystals. By keeping the DNA in the sample cell at a more modest concentration of 33 μM, these phase transitions and non-specific residual heats were avoided.

The dependence of heat evolved upon binding the DNA-binding domain with d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), and d(G₄T₄G₄) DNAs was characteristic for single-site binding reactions and consistently yielded a 1:1 stoichiometry of DNA binding (Table 1). Representative titrations and isotherms for d(T₄G₄) DNA are shown in Figure 2 (a)–(c). The d(T₄G₄T₄G₄) DNA, with two telomere repeats, has potentially two independent and equivalent protein binding sites and was expected to produce a similar isotherm with a stoichiometry parameter *n*=2. Surprisingly, we instead observed isotherms for the binding of protein with d(T₄G₄T₄G₄) DNA that show two sequential and non-equivalent binding events each with a stoichiometry parameter *n*=1 (Figure 2(d)–(f) and Table 1).

DNA is not folded into G-quartets in these experiments

DNA with telomere repeat sequences will form extremely stable four-stranded G-quartet-stabilized structures in the presence of appropriate cations.^{23–31} These G-quartet structures are likely involved with telomere biology,^{17,32–34} but in the absence of other factors these folded DNAs interfere with alpha protein binding^{35–37} and hamper the otherwise processive extension of telomeres by telomerase.^{38,39} Experiments were carried out in lithium chloride-containing solutions, and we are convinced that under these conditions G-quadruplexed DNA is at most a small component of the ensemble of DNA structures in these experiments since (1) adding potassium chloride eliminates all measurable enthalpy of DNA–protein interaction (data not shown), and (2) in the absence of added potassium ions, the measured stoichiometry of DNA–protein association is very close to one (Tables 1 and 3). If a substantial portion of the DNA formed stable G-quadruplexes, then this stoichiometry parameter would be substantially less than one, since a significant fraction of DNA would be unavailable for association with protein. Because *n*~1 in all of our calorimetric titrations, we conclude that kinetically inert G-quadruplexed DNA was not present at significant levels in our experiments.

Affinity and free energy of binding DNA

The DNA containing a single telomere repeat, d(T₄G₄), binds the alpha N-terminal DNA-binding domain at 25 °C with $K_{D-DNA}=102(\pm 6)$ nM ($\Delta G=-9.5$ kcal/mol, Table 1). Shortening the DNA by removing the 5'-terminal TT nucleotides reduces the binding affinity such that d(T₂G₄) binds protein with $K_{D-DNA}=790(\pm 50)$ nM ($\Delta G=-8.3$ kcal/mol) at 25 °C. The affinities measured for d(G₃T₄G₄) and d(G₄T₄G₄) are $K_{D-DNA}=80(\pm 5)$ ($\Delta G=-9.7$ kcal/mol) and $K_{D-DNA}=120(\pm 20)$ ($\Delta G=-9.4$ kcal/mol), respectively, indicating that adding G nucleotides to the 5'-end of the d(T₄G₄) DNA has almost no effect on the overall free energy of binding. Comparing these measures shows that an eight-nucleotide d(T₄G₄) may represent the minimal binding site, and additional nucleotides have only a marginal effect on association.

The d(T₄G₄T₄G₄) DNA possesses two binding sites, one strong site, which we will call the “A site” characterized by $K_{D-DNA(A)}=13(\pm 4)$ nM ($\Delta G=-10.8$ kcal/mol) and a weaker “B site” with $K_{D-DNA(B)}=5600(\pm 600)$ nM ($\Delta G=-7.2$ kcal/mol) at 25 °C. Comparing all of the DNAs yields the following trend for DNA–protein complex stability: A site of d(T₄G₄T₄G₄) > d(G₄T₄G₄) ≈ d(G₃T₄G₄) ≈ d(T₄G₄) > d(T₂G₄) > B site of d(T₄G₄T₄G₄), a series that spans 3.6 kcal/mol in binding free energy.

A molecular interpretation of the two binding events associated with d(T₄G₄T₄G₄) is that site A corresponds with the 3'-terminal TTTTGGGG and site B corresponds with the 3'-distal TTTTGGGG repeat. Although these sites are identical in sequence each is chemically distinct, since one ends with a 3' OH group while the other ends with a bridging phosphodiester linkage. An alternative explanation for weaker site B binding invokes site-to-site interactions. In this model protein occupancy at the first binding site influences binding at the second site. We recognize that heterogeneous sites and equivalent but interacting sites cannot be easily differentiated;⁴⁰ however, previous binding studies have shown that the alpha N-terminal domain prefers sites with a 3' OH group,¹⁹ a result we confirmed through experiments with DNA sequence variants (this work), meaning that a portion of the difference in binding affinity measured for sites A and B is due to intrinsic differences between these sites.

Enthalpy, entropy and heat capacity for DNA binding

The ITC method provides measures of enthalpy and entropy of binding reactions in addition to binding free energy and dissociation constants.^{41–43} Enthalpy and entropy reflect various interactions such as formation of van der Waals contacts, hydrogen bonds and release of water and hydrated ions as hydrophobic and polar groups become buried at the DNA–protein interface. Binding reactions with d(T₂G₄) and d(T₄G₄) DNAs were strongly exothermic, indicating a favorable negative enthalpy (ΔH) of binding (Figure 3(a) and (b)). In contrast, binding reactions with d(G₃T₄G₄), d(G₄T₄G₄), and the A site of d(T₄G₄T₄G₄) showed more favorable positive entropy (ΔS) of binding (Figure 3(a) and (c)).

The balance between enthalpy and entropy is temperature-dependent, since both ΔH and ΔS decrease monotonically as function of temperature (Figure 3(b) and (c)). In our entropy and enthalpy measures we observed strikingly systematic trends with negative enthalpy decreasing in magnitude and positive entropy increasing in magnitude as a function of DNA length (Figure 3(a)). Comparing d(T₄G₄) and d(T₄G₄T₄G₄), there was complete reversal of the relative importance of enthalpy and entropy. At 20 °C, the d(T₄G₄) binding reaction is enthalpy-driven ($\Delta H < 0$, $\Delta S \sim 0$), and the d(T₄G₄T₄G₄) binding reaction at the A site is entropy-driven ($\Delta H \sim 0$, $\Delta S > 0$). As described further in Discussion, this trend with respect to DNA length likely reflects structures in the uncomplexed DNA, which become increasingly more stable as additional G and T nucleotides are added to the 5' end of the DNA, and which are resolved upon binding DNA with protein.

We measured ΔC_p from the slope of ΔH versus temperature plots (Figure 3(b)). The DNA binding reactions were associated with negative heat capacity changes. Overall, the magnitude of heat capacity change ($|\Delta C_p|$) increased with DNA length; however, the enthalpy changes accompanying binding of d(T₂G₄) and d(T₄G₄), are significantly less temperature-dependent than seen for d(G₃T₄G₄), d(G₄T₄G₄), and the A site of d(T₄G₄T₄G₄) (Figure 3(d) and Table 2). We believe that the pattern of ΔC_p with respect to DNA length is consistent with the idea that structural changes in the DNA are coupled with binding.

The magnitude of ΔC_p is dramatically different for binding at sites A and B of d(T₄G₄T₄G₄). While binding at site A is characterized by the largest magnitude in heat capacity change with $\Delta C_p = -360(\pm 8)$ cal mol⁻¹ K⁻¹, binding at site B has the smallest magnitude of ΔC_p for all DNAs examined in this study with $\Delta C_p = -67(\pm 1)$ cal mol⁻¹ K⁻¹. These heat capacity changes suggest that binding events associated with A and B sites are different in some manner.

Polyelectrolyte effect

In order to determine the number of ions paired upon binding of DNA with protein, the association constant was measured at different concentrations of lithium chloride (Figure 4 and Table 3). Lithium (Li⁺) was chosen to avoid complications arising from kinetically inert G-quartet stabilized structures that form in the presence of other cations such as Na⁺, K⁺, and NH₄⁺.^{23,24,28} Although Li⁺ and these other ions interact very differently with the O⁶ coordination sphere of G-quartets, their hydrated forms likely interact very similarly with the polyanion phosphodiester groups of DNA.^{44,45} For instance, studies with the bacterial single strand DNA binding protein in a variety of salts showed that DNA-protein interactions were the same whether measured in solutions containing NaCl or LiCl.⁴⁵

Binding reactions for each of the single strand DNA fragments showed relatively weak dependence on salt concentration. The number of ion pairs (Z) was calculated from the slope of $\ln(K_{A-DNA})$ versus $\ln([LiCl])$ plots (Figure 4) normalized by -0.71 , the accepted value for single strand DNA⁴⁶ (Table 4). For d(T₂G₄) $Z=1.3$, whereas for d(T₄G₄) $Z=2.1$. d(G₃T₄G₄), d(G₄T₄G₄), and site A of d(T₄G₄T₄G₄) each showed a salt dependence of protein association nearly identical with that of d(T₄G₄) with $Z=2.0-2.5$. The B site of d(T₄G₄T₄G₄) was characterized by $Z=0.9$, further indication that binding events associated with site B are different from those associated with site A. It should be noted that the salt-dependence of DNA association was determined at 20 °C for the d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), and d(G₄T₄G₄) DNAs while data were obtained at 25 °C for the d(T₄G₄T₄G₄) DNA. Comparison with d(T₄G₄T₄G₄) is nevertheless reasonable, since the slope of $\ln(K_{A-DNA})$ versus $\ln([LiCl])$ plots is generally only weakly temperature-dependent,⁴⁷ except in the case of strongly polar interactions.⁴⁸

Extrapolation to 1 M LiCl provides a free energy reference point ($\Delta G_{1M LiCl}$) and allows calculation of the relative contributions of electrostatic and non-electrostatic interactions under physiological conditions of 0.15 M LiCl (Table 4). Approximately 85% of the total free energy of binding corresponds with non-electrostatic interactions for all DNAs tested (Table 4). A similarly high non-electrostatic contribution for binding telomere-derived DNA has also been observed for a telomere single strand DNA binding system from fission yeast.⁴⁹ Single strand telomere DNA binding proteins apparently adapted to recognize DNA in a sequence-specific manner by relying less on electrostatic interaction in favor of other non-electrostatic interactions such as stacking of aromatic groups and hydrogen-bonding.⁴⁹⁻⁵¹

Binding of DNA variants

The penultimate G nucleotide is critical for binding to the alpha DNA-binding domain as determined for d(TTTTGGGG)-derived DNAs.²⁰ We replaced this nucleotide with cytosine

(C) at G7 and G15 in the d(GGGTTTGGGG) DNA[†] and at G15 in d(TTTTGGGGTTTGGGG) in order to address the question of whether A and B sites correspond to particular locations on the telomere DNA fragment. Titration of the alpha N-terminal domain into d(GGGTTTGGCG) showed no detectable binding at 20 °C and 30 °C, whereas titration into d(GCGTTTGGGG) at 25 °C showed a similar thermogram and binding isotherm as seen for the cognate d(G₃T₄G₄) DNA (Figure 5(a)). The measured enthalpy, entropy, free energy of binding, and dissociation constants measured for these DNA variants are reported in Table 5. The position-dependence of the G-to-C substitution confirmed that the 3'-terminal G tract is critical for binding while the 3'-distal G tract is less important. The small differences in the magnitude of negative enthalpy and free energy of binding measured for the d(GCGTTTGGGG) likely reflects differences in the enthalpy and stability of G•C Watson-Crick *versus* G•G Hoogsteen base-pairs present in the ensemble of unbound DNA structures.

Nucleotide substitution variants of the 11-mer d(G₃T₄G₄) DNA indicated that the alpha N-terminal domain binds preferentially to sites with a 3' OH group rather than to internal sites, consistent with interactions seen in the DNA-alpha N-terminal domain co-crystal structure¹⁹ and conclusions from previous binding studies.²⁰ Since replacement of the penultimate G in d(G₃T₄G₄) completely abolished protein association, we reasoned that a similar replacement in d(T₄G₄T₄G₄) should provide a route to measure the binding properties of the 3'-distal site of d(TTTTGGGGTTTGGCG) before the 3'-terminal site becomes occupied. For comparison, d(T₄G₄T₄) DNA was evaluated, since this DNA also places the TTTTGGGG site at a 3'-distal location. Figure 5(b) compares binding isotherms obtained for d(T₄G₄T₄G₄), d(TTTTGGGGTTTGGCG), and d(T₄G₄T₄). The similarity of the first part of the binding isotherm for d(TTTTGGGGTTTGGCG) and second part of the binding isotherm for d(T₄G₄T₄G₄) and also the complete lack of binding with d(GGGTTTGGCG) suggest that there is a reversal in binding order with site B occupied first after which the mutated site A becomes occupied. Additional proof of this suggestion comes from the binding profile obtained with d(T₄G₄T₄) DNA, which shows one binding step with a $K_{D-DNA} = 1000(\pm 50)$ nM, very similar to the $K_{D-DNA} = 1400(\pm 700)$ nM measured for the first B site binding transition of d(TTTTGGGGTTTGGCG).

On the basis of these comparisons we assign the first binding event for d(TTTTGGGGTTTGGCG) to a 3'-distal site. The $K_{D-DNA(B)}$ measured for site B in the context of cognate d(T₄G₄T₄G₄) is slightly higher than measured for the 3'-distal site in d(TTTTGGGGTTTGGCG) and d(T₄G₄T₄); however, these sites are likely related. Differences in free energy of binding at site B for these different DNAs ($\Delta\Delta G \sim 1$ kcal/mol) are not unexpected given that the A site is complexed with protein in d(T₄G₄T₄G₄) but unoccupied in d(TTTTGGGGTTTGGCG) during the binding step at site B. The picture emerging indicates that interaction between the two TTTTGGGG sites makes each responsive to the binding state of the other site. Such site-to-site interactions could be mediated either through DNA structural changes or through direct protein-protein contact.

Further evidence for interaction between binding sites comes from comparison of the binding properties of the mutated sequences in d(GGGTTTGGCG) and d(TTTTGGGGTTTGGCG). As noted above, no binding was detected with the shorter DNA at either of two temperatures. Binding of d(TTTTGGGGTTTGGCG) at 25 °C is characterized by two transitions, the weaker of which we assign to an impaired A site with $K_{D-DNA(A-mutated)} = 20,000(\pm 7000)$ nM. This impaired A site was not accessible in the context of the 11-mer d(GGGTTTGGCG) DNA but is available in d(TTTTGGGGTTTGGCG) so long as the B site is bound to protein. These results are consistent with the idea that site-to-site

[†]We will refer to nucleotide positions by their number in the d(TTTTGGGGTTTGGGG) sequence.

interactions influence binding behavior at the two adjacent TTTTGGGG repeats of the d(T₄G₄T₄G₄) telomere fragment.

Comparison of d(T₄G₄T₄) and any of the other DNAs with one TTTTGGGG sequence gives an estimate for the free energy difference distinguishing binding at 3'-distal and 3'-terminal sites, $\Delta\Delta G = +1.5$ kcal/mol, corresponding to an approximately tenfold higher K_{D-DNA} at 25 °C for the 3'-distal site. Extending this estimate to the d(T₄G₄T₄G₄) telomere-derived DNA, the first binding event at site A involves binding at either the 3'-terminal telomere repeat or the 3'-distal repeat to form a DNA•protein complex with approximately 90% of the DNA occupied at the 3'-terminal site and 10% occupied at the 3'-distal site. That the second binding event at site B is associated with a less favorable free energy of binding could be a consequence of at least three effects: (1) most of the second binding event involves association with the intrinsically weaker 3'-distal site; (2) the statistical likelihood of association at the second site is less, since there is only one way to add the second protein but two ways to dissociate the protein•DNA•protein complex; and (3) the available binding site is adjacent to an occupied site and therefore involves placing two proteins close to each other.

Effects (1) and (2) work against binding the second protein, while effect (3) could either help or discourage binding depending on whether close approach of the two proteins leads to overall favorable cooperative interactions or overall unfavorable anti-cooperative interactions. The +3.6 kcal/mol free energy of DNA binding distinguishing site B from site A predicts either that a certain degree of steric strain or other repulsive terms are introduced through juxtaposition of the two alpha DNA-binding domains, or that the second protein makes a new set of interactions with DNA, which are collectively weaker than those made by the first protein, in order to avoid steric collisions. We are currently undertaking structural studies in order to distinguish between these two possibilities.

Discussion

Thermodynamic character of binding d(T₄G₄)

Experiments presented here showed that binding of the alpha N-terminal domain with a single strand telomere d(T₄G₄) repeat at 20 °C is associated with a favorable change in enthalpy ($\Delta H = -9.6$ kcal/mol) and a near zero change in entropy ($\Delta S = -0.1$ cal mol⁻¹ K⁻¹). The temperature dependence of ΔH indicated a modest and negative change in heat capacity ($\Delta C_p = -169$ cal mol⁻¹ K⁻¹), while the salt dependence of DNA association showed that most of the free energy is derived from non-electrostatic interactions at physiological salt concentrations. The thermodynamic character of d(T₄G₄)-alpha N-terminal domain association can now be compared with interactions seen in co-crystal structures containing the alpha N-terminal domain and DNA.^{19,52} These structures revealed four classes of interactions: (1) arginine-phosphate ion pairs; (2) π - π tyrosine-base stacking interactions; (3) hydrogen bonds between base edges and polar carboxylate and amide bearing amino acid residues; and (4) hydrophobic type interactions between moieties of the deoxyribose and apolar side-chains. Here we discuss how these interactions may contribute to the thermodynamics of DNA-protein binding measured in our experiments.

The co-crystal structures show two phosphate groups of d(T₄G₄) making salt-bridges with two arginine residues^{19,52} that likely contribute to saltsensitive DNA-protein contacts totaling ~15% of the overall free energy of DNA binding at 0.15 M lithium chloride. Stacking interactions between bases of the DNA and four tyrosine residues of the protein complement salt-bridges seen in the cocrystal structures. An additional base makes a similar stacking-type interaction with the guanidinium group of an arginine residue for a total of five such π - π contacts at the DNA-protein interface. Model compound studies⁵³ and protein-DNA examples⁵⁴ showed that stacking of aromatic residues is associated with favorable changes in

enthalpy. Given the number of these types of contacts, the stacking of aromatic and heteroaromatic groups may be a dominant force leading to a stable single strand DNA–protein complex.

Telomere single strand DNA binding proteins have evolved to recognize a particular sequence of DNA, and some of the interactions seen in alpha N-terminal domain containing co-crystal structures involve apparently sequence-specific hydrogen bonds between the edge groups of DNA bases and polar aspartate, asparagine, arginine, and glutamine side-chains of the protein.^{14,19,52} H-bond formation and removal of polar groups from water are enthalpy-driven processes,^{55–57} so these DNA sequence-specific interactions should augment the favorable enthalpy term of DNA–protein complex formation.

Hydrophobic-type contacts are seen at the DNA–protein interface where aliphatic moieties of the DNA, especially the deoxyribose group, nestle with valine, isoleucine, and phenylalanine residues of the protein. These types of contacts with the 2'-methylene group likely contribute to DNA *versus* RNA discrimination.^{14,19,52} Removing hydrophobic groups from solvent is entropy-driven.^{58–60} However, there are fewer of these hydrophobic interactions in comparison with the enthalpy-driven aromatic stacking and hydrogen-bonding interactions, consistent with the thermodynamic signature determined here showing that enthalpy favors DNA–protein complex formation while the net entropy change coupled with d(T₄G₄) binding is close to zero.

The interactions seen in the *O. nova* telomere end binding protein–single strand DNA complexes are different in detail, but similar in general terms to interactions seen in other telomere single strand DNA–protein complexes from human and fungi. The human and fission yeast protection of telomere protein (POT1) DNA binding domain each share one folding unit with the *O. nova* alpha N-terminal domain,^{61,62} and each protein makes hydrogen bonding contacts with sequence-specific groups of the DNA, stacking interactions involving nucleotide bases and aromatic residues, and a few salt-bridges with DNA phosphate groups.^{61,62} The single strand telomere DNA binding domain from the CDC13 protein of budding yeast, is constructed from a structurally similar fold.⁶³ A scanning alanine mutagenesis study for the CDC13 single strand telomere DNA binding domain confirmed and highlighted the importance of aromatic side-chains for DNA affinity.⁵⁰ The repeated use of similar types of interactions in these telomere single strand DNA binding proteins likely reflects a common evolutionary pressure to recognize and bind with the telomere repeat in a sequence-specific manner.^{15,50}

Structural changes coupled with binding DNA

The 8 nt d(T₄G₄) DNA represents only a portion of the 16 nt single strand d(T₄G₄T₄G₄) DNA found at the ends of mature telomeres in *O. nova*. During *de novo* telomere addition and telomere replication the single strand DNA may be even longer.^{64,65} In order to understand how longer DNAs interact with the alpha DNA-binding domain, we compared the thermodynamic parameters characterizing binding the short DNAs, d(T₂G₄) and d(T₄G₄), with those for binding d(G₃T₄G₄), d(G₄T₄G₄) and d(T₄G₄T₄G₄). The results were consistent with the idea that nucleotides added to the 5' end of a TTTTGGGG repeat likely interact only weakly, if at all, with protein. Enthalpy and entropy changes accompanying protein association correlate in a systematic manner to DNA length, however. To explain this pattern required careful consideration of the unbound DNA structure and changes in DNA structure coupled with DNA–protein association.

Several lines of experimental evidence suggest that single strand DNA in solution retains a large degree of organized structure built around base-base stacks. Calorimetric studies of structural transitions in oligo-A indicated that purine bases in a single strand RNA stack with a substantial enthalpy of -3 kcal/(mol base stack).^{66,67} Other calorimetric analyses of 13 and 14 –base-pair DNAs containing dG•dC and dA•dT base-pairs, concluded that a substantial

portion of enthalpy in duplex DNA is preserved in the dissociated single strands in the form of ordered helical base stacked structure. 55•68 Accordingly, in Figure 6(a) the uncomplexed form of d(T₄G₄) is sketched in one hypothetical form closely resembling a strand of B-form DNA with bases making base-stacking interactions. We realize that free in solution, the single strand DNA may adopt a range of different conformations, with differing degrees of base stacking. However, the model highlights the point that single strand DNA is something other than a “random coil” ensemble and further illustrates that DNA–protein association is associated with bases unstacking⁶⁹ (Figure 6(c)).

The potential for forming organized structure is even greater in the longer DNAs, d(G₃T₄G₄) d(G₄T₄G₄) and d(T₄G₄T₄G₄), since the additional GGG or GGGG tracts can form intramolecular G•G base-pairs with the 3'-terminal GGGG tract. A hypothetical model for d(G₄T₄G₄) that is consistent with spectroscopic evidence is shown in Figure 6(b). The d(G₄T₄G₄) molecule has been studied in lithium chloride by NMR,⁷⁰ and under low concentrations of sodium chloride by hydrogen-deuterium exchange, Raman spectroscopy, and gel electrophoresis.²¹ These studies indicated the DNA forms a hairpin structure with Hoogsteen G•G base-pairs.^{21,70} In moderate concentrations of Na⁺, K⁺ or NH₄⁺, d(G₄T₄mG₄) forms related but different structures with two molecules associating in G-quartet stabilized dimers.^{30,71,72} Recent experiments in a closely related ciliate, *Stylonychia lemnae*, provide compelling evidence that telomere end binding proteins interact with such structures *in vivo*.¹⁷

Binding the d(G₃T₄G₄), d(G₄T₄G₄) and d(T₄G₄T₄G₄) DNAs could be coupled to dissociation of G•G Hoogsteen base-pairs in addition to base unstacking. Formation of G•G Hoogsteen base-pairs is an enthalpy-driven process compensated by an unfavorable decrease in entropy. Differential scanning calorimetry with triplexed^{73–77} and quadruplexed⁷⁸ DNAs measured fairly consistent values of $\Delta H = 3–5 \text{ kcal mol}^{-1}$ and $\Delta S = 9 \text{ cal mol}^{-1} \text{ K}^{-1}$ for Hoogsteen base-pair dissociation. Resolving G•G base-pairs should therefore contribute an unfavorable positive enthalpy and favorable positive entropy to the binding reaction, exactly as is observed comparing d(G₄T₄G₄), d(G₄T₄G₄), and the A site of d(T₄G₄T₄G₄) with d(T₄G₄) (Figure 3(a)). The more favorable positive entropy change characterizing binding reactions of these longer DNAs likely derives from increased flexibility in the 5'-terminal nucleotides along with interactions of these nucleotides with solvent.

In addition to G•G base-pairs, d(T₄) nucleotides shape the structure of d(G₃T₄G₄), d(G₄T₄G₄) and d(T₄G₄T₄G₄). Evidence that d(T₄) loops form ordered structures comes from studies of Watson–Crick base-paired hairpin DNAs and G-quartet stabilized DNA dimers. In the context of a Watson–Crick base-paired hairpin DNA, d(T₄) loops augmented thermal stability of the duplex to a greater degree than d(C)₄, d(G)₄, or d(A)₄.^{79,80} NMR and X-ray studies confirmed that the d(T₄) nucleotides within such loops are highly organized.^{81–84} Within G-quartet stabilized DNA dimers, d(T₄) nucleotides connect diagonally opposed GGGG tracts. The same d(T₄) structure, with T–T and T–G base stacking, was observed in several crystal forms^{31,85} and in solution by NMR,^{28,86} further indication that d(T₄) nucleotides have an intrinsic propensity for forming structure. Since d(T₄) loops adopt organized structures in several different contexts, we believe that the d(T₄) loop of hairpins is structured in d(G₃T₄G₄), d(G₄T₄G₄) and d(T₄G₄T₄G₄) DNAs and that protein binding reactions are coupled with breaking this d(T₄) structure.

These proposed structural changes in the DNA may be accompanied by subtle adjustments in the structure of the alpha N-terminal domain coupled with binding DNA. The N-terminal domain is constructed from two oligonucleotide/oligosaccharide/oligopeptide binding folds^{14,87} (OB folds). Figure 6(d) shows the superposed alpha carbon traces of these two OB folds from uncomplexed and DNA-bound forms of the N-terminal domain. Overall, the

structures are very similar with a RMS deviation of less than 0.4 Å for all alpha carbons. As noted previously,¹⁹ atom displacements within each OB fold are concerted, however, so that OB fold-1 and OB fold-2 rotate slightly with respect to each other. This subtle relative movement of OB folds is more easily detected in a two-frame movie (Movie S1), or as intramolecular distance differences that show certain loops have shifted by 1.8 Å comparing the uncomplexed and DNA-bound proteins. Although these DNA-binding induced shifts in atom positions are small, they may trigger more dramatic changes in structure of the alpha protein that subsequently lead to protein–protein association between alpha and beta subunits in the full *O. nova* telomere end binding protein. Such an allosteric mechanism could potentially explain cooperativity between DNA binding and protein–protein association uncovered in earlier studies of the *O. nova* telomere end binding protein¹³ and recently described quantitatively in the form of a thermodynamic binding cycle.¹⁸

Heat capacity change of DNA–protein binding

The structural changes coupled to DNA–protein binding proposed above could contribute to the observed change in heat capacity (ΔC_p) associated with binding reactions. It is widely recognized that heat capacity is an important thermodynamic property intrinsic to folding and binding of macro-molecules, yet the molecular basis for changes in heat capacity is still not well understood.⁸⁸⁻⁹⁰ A negative ΔC_p is considered a hallmark for sequence-specific *versus* non-sequence-specific recognition of double-stranded DNA.^{59,91-93} Single strand DNA is less restrained and likely samples more conformations compared with double-stranded DNA. Since heat capacity depends critically on the degeneracy of states accessible within a molecular ensemble,⁹⁰ trends observed for binding double-stranded DNA may or may not apply generally to binding single strand DNA. The present work provides examples of ΔC_p measured for the sequence-specific recognition of telomere-derived single strand DNAs.

A modest negative change in heat capacity ($\Delta C_p < 0$) characterizes association of the alpha N-terminal domain with each of the DNAs, and the magnitude of this negative heat capacity change ($|\Delta C_p|$) increases with DNA length (Figure 3(d)). The DNA length dependence of $|\Delta C_p|$ is not uniform, however, with a significantly larger $|\Delta C_p|$ associated with the binding reactions of longer DNAs, d(G₃T₄G₄), d(G₄T₄G₄) and site A of d(T₄G₄T₄G₄), compared with those of the shorter DNAs, d(T₂G₄) and d(T₄G₄). The simplest interpretation is that, along with base unstacking,⁶⁹ dissociation of intramolecular G•G base-pairs contributes to $|\Delta C_p|$. The heat capacity change measured for DNA strand dissociation^{55,94-98} is positive, and thus apparently inconsistent with the idea that G•G dissociation should lead to a more negative heat capacity change. However, since base unstacking and G•G dissociation processes are coupled with protein binding, an “apparent” negative ΔC_p term is anticipated,^{69,90} exactly as measured for the binding reactions of d(G₃T₄G₄), d(G₄T₄G₄) and the A site of d(T₄G₄T₄G₄). The same kind of structural transitions may explain the very large and negative heat capacity change observed for binding of G-rich DNA by F factor relaxase.⁹⁹ Larger than expected negative heat capacity changes for DNA-binding events have often been discussed in terms of changes in protein structure coupled with binding.⁹⁹⁻¹⁰² Structural changes in single strand DNA coupled with binding can also contribute to the final value of ΔC_p .^{69,103,104} The heat capacity changes measured in our system are consistent with the idea that binding DNA is accompanied by base unstacking and base-pair dissociation in the telomere single strand DNA.

Biological function and evolution of the telomere end binding protein

Although the molecular picture is far from complete it is important to consider how the protein binding events measured here relate to subsequent interactions essential for the biology of telomeres. The thermodynamic binding properties of alpha and its N-terminal domain described here and in previous *in vitro* studies¹⁸ show how alpha might participate in loading the 3'-terminal single strand DNA into a productive complex with telomerase by resolving G•G

base-paired structures (Figure 7). Because telomerase from *O. nova* is unable to initiate extension for folded forms of the single strand DNA,³⁸ and formation of the alpha-DNA complex is incompatible with these folded structures, protein binding can maintain the DNA in an extended conformation required for productive interaction with telomerase. Froelich-Ammon *et al.* observed that formation of an alpha•DNA•alpha complex diminished telomerase activity, whereas an alpha•DNA complex served as a suitable primer for telomerase-mediated DNA extension.¹⁰⁵ In the closely related ciliate *S. lemnae*,¹⁷ alpha is important for attachment of DNA to the nuclear envelope and for regulation of DNA structures that are immunoreactive with G-quartet-recognizing antibodies.¹⁷ These results demonstrate an active role for alpha in forming and resolving DNA structures in a manner timed with DNA replication.¹⁷ According to our measures, given a limited amount of alpha the 3'-terminal TTTTGGGG binding site will be occupied by protein leaving the 3'-distal nucleotides exposed and thus free to interact with telomerase.

If alpha binds preferentially to the 3'-terminal site, how does telomerase gain access to the 3' OH group required for nucleotidyl transfer and DNA extension? The single strand DNA fragments that we have studied only approximate complete telomeres, and the presence of a complementary DNA strand present in authentic telomere DNA may guide the alpha protein away from the 3'-end. Alternatively, as suggested for human telomere binding protein POT1,¹⁰⁶ there may be other cellular factors that move the end-specific protein from a 3'-terminal site to an internal binding site. In the full *O. nova* telomere complex, beta, the second telomere protein, could be a key player in this interconversion event. Upon association with this second protein subunit, the 3'-terminal TTTTGGG portion of the DNA makes new interactions with a binding pocket created by juxtaposition of alpha and beta subunits. Dissociation of beta, potentially triggered by phosphorylation,^{17,107} would leave the 3'-terminal region exposed and ready to interact with telomerase.

During *de novo* telomere synthesis and telomere replication, the 3'-terminal single strand DNA is extended well past the 16 nt length found at mature telomeres.^{64,65,108,109} Binding to 3'-distal repeats as observed during the second binding transition associated with site B may resemble the function of an ancestral, non-sequence-specific single strand DNA binding protein from which the telomere end binding protein was derived. All organisms have such a non-sequence-specific single strand DNA binding protein that acts at the replication fork to maintain separated strands in a form suitable to act as template for DNA polymerase during DNA synthesis.^{110,111} The crystal structure of the human replication protein A single strand DNA binding domain¹¹² revealed two OB folds⁸⁷ tandemly arrayed to interact with eight nucleotides of single strand DNA. In bacteria the ssDNA binding protein is a homo-tetramer, also constructed from multiple OB folds.¹¹³ The N-terminal domain of alpha comprises two such OB folds.¹⁴ Structural homology among these proteins lends support to the idea that high affinity, sequence-specific recognition of 3'-terminal single strand DNA evolved through gene duplication and divergence.^{14,15} The site B interactions described here further suggest that interaction with non-terminal telomere single strand DNA was retained even as the protein became more specialized at capping the very ends of telomeres.

Experimental Methods

Protein and DNA purification

Alpha (aa 1–495) and alpha N-terminal domain (aa 1–326) from the *O. nova* telomere end binding protein were expressed in *Escherichia coli* BL21 (DE3) pLysS and purified as described,^{14,18} except that the final size-exclusion chromatography was developed in binding buffer consisting of 0.15 M lithium chloride and 0.05 M Tris (pH 7.5). Protein concentration was determined with the use of absorbance at 280 nm and extinction coefficients calculated according to the number of tyrosine and tryptophan residues in each protein.¹¹⁴

Trityl-group protected DNA oligonucleotides were synthesized by the University of Utah DNA/peptide research core facility as multiple 1 μ mol batches and resuspended in HPLC buffer A (10 mM triethylamine ammonium acetate (pH 6.0)). Protected oligonucleotides were purified by reverse phase HPLC using 40 min linear gradients from 14%–22% HPLC buffer B (10 mM triethylamine ammonium acetate (pH 6.0), 75% acetonitrile). The trityl protecting group was removed by resuspending the lyophilized material with 10 ml of 80% acetic acid. Reactions were incubated at room temperature for 20 min and then quenched with 50 ml of ice cold water followed by lyophilization. Deprotected DNAs were resuspended in HPLC buffer A, insoluble material was removed by filtration through a 0.2 μ m PVDF low-protein binding filter (Millipore), and a second reverse phase HPLC purification was developed using a 30 min linear gradient from 5%–10% HPLC buffer B. Solvents were removed by repeatedly resuspending lyophilized material with 10 ml of water. Pure DNA oligonucleotides were resuspended with a minimal volume of binding buffer and dialyzed overnight against $\times 1000$ volumes of binding buffer. DNA concentrations were determined with use of absorbance at 260 nm and molar extinction coefficients calculated using the nearest neighbor model.¹¹⁵

Since sodium ions promote the formation of kinetically inert G-quartet stabilized DNA quadruplexes while lithium ions do not stabilize G-quartets, calorimetry experiments described here were conducted in lithium chloride-containing solutions and reasonable precautions were taken to exclude potassium or sodium ions. For instance, EDTA was purchased as the free acid and titrated with Tris base, not sodium hydroxide, and the pHs of buffered solutions were measured in separate aliquots to avoid contact with a potassium chloride equilibrated electrode.

Native gel electrophoresis

7.6% (w/v) acrylamide:0.2% bis-acrylamide gels were cast in a 12 cm $\times$ 14 cm horizontal tray with *n*-butanol layered on top to promote uniform polymerization. The 89 ml gel mixture contained 10% 0.25 $\times$ TBE running buffer. Polymerization proceeded for 2 h. The gel was submerged in 0.25 $\times$ TBE (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA) and pre-run for 30 min at 110 mV and +4 $^{\circ}$ C. 10 μ l samples were loaded in each lane and electrophoresis proceeded at 110 mV for an additional 4 h or 5 h, respectively, for alpha N-terminal domain and full-length alpha subunit. The concentration of protein in each sample was 110 μ M for alpha N-terminal domain or 80 μ M for the full-length alpha subunit.

Isothermal titration calorimetry

Isothermal titration calorimetry was performed with use of a MicroCal VP-ITC instrument. To prepare binding components, DNA and protein solutions were loaded into separate 2.5 ml, 1000 molecular weight cutoff dialysis units (Amersham Biosciences) and dialyzed at +4 $^{\circ}$ C overnight against one common 500 ml volume of binding buffer. The DNA was diluted to 30 μ M, degassed, and placed in the calorimeter sample cell. For titration of all DNAs except d (T₄G₄T₄G₄), the protein was diluted to 330 μ M, degassed, and placed in the titration syringe. For titration of d (T₄G₄T₄G₄), the protein concentration was adjusted to 540 μ M. All dilutions were prepared by adding the equilibrating solution obtained after dialysis was complete. During the experiment, the sample cell was stirred at 300 rpm. Titration of all DNAs except d (T₄G₄T₄G₄) started with one initial 3- μ l injection followed by 30 8- μ l injections spaced by 200-s intervals. Titration of d (T₄G₄T₄G₄) started with one initial 2- μ l injection followed by 41 6- μ l injections.

Heat evolved or absorbed upon each injection was quantified by integration of the power *versus* time plot corrected for the heat of injecting buffer alone into the sample solution. Enthalpy (ΔH), dissociation constant (K_{D-DNA}), entropy (ΔS), and stoichiometry of binding (*n*) were obtained by non-linear least-squares fitting of the heat *versus* protein:DNA data to

one-site or two-site models provided with the data analysis software (Origin). Free energy of binding (ΔG) was calculated from Gibb's equation $\Delta G = \Delta H - T\Delta S$.

Calculation of the relative proportion of non-electrostatic free energy at 0.15 M lithium chloride

Plots of $\ln(K_{A-DNA})$ versus $\ln([LiCl])$ defined by four measurements were extrapolated to obtain the free energy of DNA association at 1 M LiCl, $\Delta G_{1M LiCl}$. At this reference point, free energy of DNA association can be assigned to non-electrostatic interactions arising from hydrogen bonds, van der Waals contacts, and hydrophobic interactions plus the energy of each ion pair at standard conditions. To obtain the relative contributions of electrostatic and non-electrostatic interactions at physiological salt concentration of 0.15 M LiCl, we applied equations (1) and (2):

$$\Delta G_{0.15 M LiCl} = \Delta G_{nonelectrostatic} + \Delta G_{electrostatic} \quad (1)$$

$$\Delta G_{nonelectrostatic} = \Delta G_{1M LiCl} - Z\Delta G_{ionpair} \quad (2)$$

where $\Delta G_{ion-pair}$ describes the energetic contribution of a lysine/arginine-phosphate ion pair under standard state conditions estimated at +0.18 kcal/mol ion pair,^{46,116-118} and Z is the number of such ion pairs determined from the slope of $\ln(K_{A-DNA})$ versus $\ln([LiCl])$ data.

Building model structures of d(T₄G₄) and d(G₄T₄G₄)

Models of single strand DNA structures free in solution were constructed using coordinates for *B*-form DNA (PDB ID 9BNA)119,120 and antiparallel/parallel stranded G-quadruplex DNA (PDB ID 1JB785). Extra strands were deleted and the sequence of each DNA was altered to be consistent with the d(T₄G₄) repeat of *O. nova* telomeres. In the case of hairpin structures, the connecting d(T₄) loop was repositioned in *O*¹²¹ to splice base-paired GGGG tracts into one DNA strand. Structures were energy minimized with CNS¹²² using updated DNA-RNA parameters,¹²³ and additional restraints describing hydrogen bonds between G•G base-pairs.

The structure of d(T₄G₄) DNA as complexed with alpha N-terminal domain was obtained from examination of three crystal structures: d(T₂G₄) in complex with alpha N-terminal domain¹⁹; d(T₄G₄) in complex with alpha₅₂ and d(T₄G₄T₄G₄) complexed with the DNA-binding portions of alpha and beta subunits (M.P.H., unpublished results). The DNA in all three examples are super-imposable for internal G nucleotides of d(T₄G₄T₄G₄) and 3'-terminal G nucleotides of d(T₂G₄) and d(T₄G₄). Details concerning the 5'-terminal nucleotides vary slightly among the structures. Our current model, for the 5'-TTTT portion, is consistent with photo-crosslinking experiments, conducted in solution, which revealed a close contact between Tyr142 of alpha and the nucleotide at position 2 in d(T₄G₄T₄G₄).¹²⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ITC	isothermal titration calorimetry
nt	nucleotides
aa	amino acids
PDB	Protein Data Bank

Figure 1.

Non-denaturing protein gel electrophoresis mobility assay. The *Oxytricha nova* telomere end binding protein alpha subunit ((d)–(f)) and its N-terminal DNA-binding domain ((a)–(c)) were incubated with increasing amounts of telomere DNA at the following DNA:protein stoichiometric ratios: 0.125:1, 0.25:1, 0.5:1, 0.75:1, 1:1, 1.5:1, and 2:1 before separating on a 7.8% acrylamide gel. The 1:1 binding reaction is indicated with a gray line. Gels were stained with Coomassie blue to reveal protein and protein-associated species. Band *a* corresponds to unbound protein, band *b* corresponds to a DNA•protein complex. The addition of d (T₄G₄T₄G₄) to alpha or the alpha N-terminal domain resulted in formation of an additional species assigned as a protein•DNA•protein complex (band *c*, in (c) and (f)).

Figure 2.

Representative ITC data. Thermograms (upper half of each panel) and binding isotherms (bottom half of each panel) obtained for the binding of alpha N-terminal domain with d(T₄G₄) ((a)–(c)) and d(T₄G₄T₄G₄) ((d)–(f)) at 10, 25, and 30 °C. A total of 31 and 42 injections were performed respectively for d(T₄G₄) and d(T₄G₄T₄G₄). Non-linear leastsquares fit of the d(T₄G₄) data with a “one set of site” model indicated one binding site with a stoichiometric parameter $n=1$. Non-linear least-squares fit of the d(T₄G₄T₄G₄) data with a “two set of sites” model indicated two binding sites with a stoichiometric parameter $n=1$ for each site.

Figure 3.

Calorimetric characterization of DNA binding. (a) Thermodynamic parameters for binding d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), d(G₄T₄G₄) and sites A and B of d(T₄G₄T₄G₄) at 20 °C are shown with free energy change (ΔG) indicated with a gray bar, enthalpy change (ΔH) with a white bar, and entropy change ($-T\Delta S$) with a black bar. The enthalpic and entropic contributions to free energy show systematic trends with $-T\Delta S$ increasingly favorable for d(G₃T₄G₄), d(G₄T₄G₄) and site A of d(T₄G₄T₄G₄). (b) Binding enthalpy change for d(T₂G₄) (▲), d(T₄G₄) (▼), d(G₃T₄G₄) (■), d(G₄T₄G₄) (◆), site A of d(T₄G₄T₄G₄) (●), and site B of d(T₄G₄T₄G₄) (○) is plotted as a function of temperature. Model lines represent linear least-squares fitting of the data to $\Delta C_p (T - T_{\Delta H=0})$, where the slope defines ΔC_p , the change in heat capacity, and the x -intercept defines $T_{\Delta H=0}$, the temperature at which enthalpy change is zero. (c) Entropy change is plotted as a function of temperature. Model curves represent non-linear least-squares fitting of the data to $\Delta C_p \ln(T/T_{\Delta S=0})$ where $T_{\Delta S=0}$ is the temperature at which entropy change is zero. Values of ΔC_p , $T_{\Delta H=0}$ and $T_{\Delta S=0}$ are reported in Table 2. (d) Heat capacity change is plotted as a function of DNA length for d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), d(G₄T₄G₄) and site A of d(T₄G₄T₄G₄). The line connects data points. The broken line highlights a sharp increase in the magnitude of ΔC_p experienced upon addition of a second G tract. As discussed in the text, the second G tract allows for the formation of intramolecular hairpin structure stabilized by G•G base-pairs that, if formed, must dissociate upon binding with protein.

Figure 4.

Salt dependence of DNA binding. Natural log-log plots of association binding constant (K_{A-DNA}) versus lithium chloride concentration were fit by linear regression. Symbols for each DNA are the same as in Figure 3. The slopes ($\partial \ln K_a / \partial \ln [\text{LiCl}]$) define $-Z\psi$, where Z is the apparent number of ion pairs formed between DNA and protein and ψ is the theoretical number of cations released into solution upon formation of one ionic pair. These parameters describing the poly-electrolyte effect are reported in Tables 3 and 4. Note that data for d(T₂G₄), d(T₄G₄), d(G₃T₄G₄), d(G₄T₄G₄) DNAs were obtained at 20 °C, while the data for sites A and B of d(T₄G₄T₄G₄) were obtained at 25 °C.

Figure 5.

Binding isotherm profiles for nucleotide substitution variants. (a) Titration of GGGTTTTGGGG (□) and GCGTTTTGGGG (Δ) at 25 °C showed high similarity in binding, whereas titration of GGGTTTTGCG (○) at 20 or 30 °C showed no detectable binding. (b) A comparison of isotherms performed at 25 °C is shown for the DNA-binding domain binding with TTTTGGGGTTTTGGGG (□), TTTTGGGGTTTTGGCG (○) and TTTTGGGGTTTT (●). The single binding site observed for TTTTGGGGTTTT has a similar K_{D-DNA} compared with site B' of TTTTGGGGTTTTGGCG and site B of TTTTGGGGTTTTGGGG (see Table 5).

Figure 6.

Structural models for telomere DNA and the alpha Nterminal domain. (a) d(T₄G₄) in a conformation characterized by base-base stacking interactions. (b) Model of d(G₄T₄G₄) in a hairpin conformation stabilized by G•G base-pairs. (c) d(T₄G₄) as modelled in complex with protein. The position of the 5'-most nucleotide is not well determined by contact with protein, however the positions of the remaining nucleotides are clearly seen in electron density maps calculated for alpha N-terminal domain containing crystals^{19,52} and crystals of d(T₄G₄T₄G₄) complexed with the DNA-binding portions of alpha and beta (M.P.H., unpublished results). Note how bases are largely unstacked in the DNA-protein complex. (d) Superposition of OB folds contained within the alpha subunit N-terminal domain for the uncomplexed protein and for the DNA-bound protein. Coordinates for the two conformations were obtained from chains C and B of PDB ID 1K8G.¹⁹ OB fold 1 comprises residues 52–149. OB fold 2 comprises residues 225–299. DNA appears to induce a subtle repositioning of OB folds. These subtle movements are best discerned in a movie available online (Movie S1).

Figure 7.

Model for interaction of the *O. nova* alpha N-terminal domain with telomere DNA. In the uncomplexed state (i), telomere DNA is represented by an ensemble with some members containing structures stabilized by G•G basepairing. The alpha N-terminal domain (square shape) binds extended DNA molecules to form an initial DNA–protein complex (ii). This binding event resolves intramolecular DNA interactions, including base-pairing and base stacking interactions. Association with protein at the second site results in a DNA–protein complex with two DNA-binding domains associated with one DNA (iii).

Thermodynamic parameters describing telomere-derived single strand DNA binding as a function of temperature at 0.15 M LiCl

Table 1

DNA	T (°C)	ΔH (kcal/mol)	ΔS (cal/(mol K))	ΔG (kcal/mol)	K_{D-DNA} (nM)	n^a
TTGGGG	10	-6.03±0.03	8.1±0.1	-8.32±0.03	380±20	1.05
TTGGGG	20	-7.25±0.05	4.0±0.2	-8.42±0.04	540±40	1.05
TTGGGG	25	-8.01±0.06	1.0±0.2	-8.32±0.04	790±50	1.05
TTGGGG	30	-8.64±0.06	-0.6±0.2	-8.44±0.04	820±50	1.03
TTTTGGGG	10	-7.81±0.02	5.9±0.1	-9.48±0.04	48±3	1.04
TTTTGGGG	20	-9.63±0.04	-0.1±0.2	-9.60±0.04	69±5	1.00
TTTTGGGG	25	-10.43±0.01	-3.0±0.1	-9.53±0.03	102±6	0.98
TTTTGGGG	30	-11.17±0.03	-5.3±0.1	-9.56±0.02	127±4	1.01
GGGTTTGGGG	10	-1.96±0.02	25.8±0.6	-9.3±0.2	70±20	1.07
GGGTTTGGGG	20	-5.06±0.02	16.1±0.1	-9.78±0.03	51±3	1.03
GGGTTTGGGG	25	-6.65±0.02	10.1±0.1	-9.66±0.04	80±5	1.08
GGGTTTGGGG	30	-8.12±0.02	6.0±0.1	-9.92±0.03	71±4	1.05
GGGGTTTGGGG	20	-2.68±0.04	23.1±0.5	-9.4±0.1	90±20	1.03
GGGGTTTGGGG	25	-4.05±0.03	18.0±0.2	-9.42±0.06	120±20	1.01
GGGGTTTGGGG	30	-5.61±0.03	15.1±0.3	-10.19±0.08	44±6	0.99
GGGGTTTGGGG	35	-7.49±0.04	8.2±0.2	-10.02±0.05	79±7	1.03
(A) TTTTGGGGTTTGGGG	10	+3.71±0.04	48.8±0.4	-10.1±0.1	16±3	0.97
(A) TTTTGGGGTTTGGGG	25	-1.58±0.04	30.8±0.6	-10.8±0.2	13±4	0.94
(A) TTTTGGGGTTTGGGG	27	-2.32±0.03	27.9±0.6	-10.6±0.2	16±5	1.01
(A) TTTTGGGGTTTGGGG	30	-3.59±0.03	25±2	-11.1±0.5	10±8	0.88
(B) TTTTGGGGTTTGGGG	10	-4.3±0.2	10.1±0.8	-7.13±0.09	3200±500	1.01
(B) TTTTGGGGTTTGGGG	25	-5.3±0.2	6.4±0.7	-7.18±0.06	5600±600	1.03
(B) TTTTGGGGTTTGGGG	27	-5.4±0.2	6.0±0.7	-7.19±0.05	5800±500	1.06
(B) TTTTGGGGTTTGGGG	30	-5.6±0.2	5.3±0.7	-7.23±0.06	6100±600	1.00

^aUncertainty in the parameter n was, on average, 0.005 and ranged from 0.001–0.02, for all data excluding site B of d(T₄G₄T₄G₄). For measures related to site B of d(T₄G₄T₄G₄), uncertainty in n was 0.03 for all measures.

Table 2

Temperature dependence of enthalpy and entropy changes in 0.15 M LiCl

DNA	$\Delta C_p^{\text{observed}}$ (cal/(mol K))	$T_{\Delta H=0}$ (K)	$T_{\Delta S=0}$ (K)
TTGGGG	-131±4	237±2	301±1
TTTTGGGG	-169±6	236±3	293±1
GGGTTTTGGGG	-309±3	277±1	309±1
GGGGTTTTGGGG	-320±20	285±1	318±2
(A) TTTTGGGGTTTTGGGG	-360±8	293±1	325±1
(B) TTTTGGGGTTTTGGGG	-67±1	219±1	327±1

Table 3

Thermodynamic parameters describing telomere-derived single strand DNA binding as a function of salt concentration

DNA	T (°C)	[LiCl] (M)	ΔH (kcal/mol)	ΔS (cal/(mol K))	ΔG (kcal/mol)	K_{D-DNA} (nM)	n^d
TTGGGG	20	0.015	-7.94±0.04	4.9±0.2	-9.38±0.03	102±5	0.85
TTGGGG	20	0.050	-7.92±0.06	3.8±0.4	-9.03±0.09	180±30	0.98
TTGGGG	20	0.150	-7.25±0.05	4.0±0.2	-8.42±0.04	540±40	1.05
TTGGGG	20	0.300	-7.07±0.08	2.8±0.4	-7.89±0.08	1400±200	0.94
TTTTGGGG	20	0.050	-8.98±0.03	4.5±0.2	-10.30±0.05	21±2	1.02
TTTTGGGG	20	0.150	-9.63±0.04	-0.1±0.2	-9.60±0.04	69±5	1.00
TTTTGGGG	20	0.300	-9.36±0.06	-1.9±0.3	-8.80±0.04	270±20	1.02
TTTTGGGG	20	0.450	-10.06±0.01	-6.0±0.9	-8.3±0.3	600±300	0.99
GGGTTTGGGG	20	0.050	-4.93±0.06	17.8±0.6	-10.1±0.2	28±8	1.00
GGGTTTGGGG	20	0.150	-5.06±0.02	16.1±0.1	-9.78±0.03	51±3	1.03
GGGTTTGGGG	20	0.300	-5.11±0.03	12.2±0.1	-8.68±0.02	330±10	0.94
GGGTTTGGGG	20	0.450	-4.71±0.03	12.8±0.1	-8.46±0.02	480±20	1.07
GGGGTTTGGGG	20	0.100	-2.32±0.03	25.5±0.4	-9.8±0.1	50±10	0.93
GGGGTTTGGGG	20	0.150	-2.68±0.04	23.1±0.5	-9.4±0.1	90±20	1.03
GGGGTTTGGGG	20	0.300	-2.23±0.04	21.8±0.4	-8.6±0.1	380±70	0.98
GGGGTTTGGGG	20	0.450	-1.73±0.05	22.2±0.6	-8.2±0.2	700±200	1.11
(A) TTTTGGGGTTTGGGG	25	0.015	-2.21±0.05	34.2±0.8	-12.4±0.2	1.0±0.4	0.98
(A) TTTTGGGGTTTGGGG	25	0.050	-1.68±0.05	32.9±0.5	-11.5±0.1	4±1	1.00
(A) TTTTGGGGTTTGGGG	25	0.150	-1.58±0.04	30.8±0.6	-10.8±0.2	13±4	0.94
(A) TTTTGGGGTTTGGGG	25	0.200	-1.29±0.06	29.0±0.8	-9.9±0.2	50±20	0.93
(B) TTTTGGGGTTTGGGG	25	0.015	-7.9±0.1	0.2±0.4	-7.92±0.03	1600±100	1.03
(B) TTTTGGGGTTTGGGG	25	0.050	-6.3±0.2	4.5±0.8	-7.61±0.06	2600±300	1.03
(B) TTTTGGGGTTTGGGG	25	0.150	-5.3±0.2	6.4±0.8	-7.18±0.06	5600±600	1.03
(B) TTTTGGGGTTTGGGG	25	0.200	-5.4±0.6	5±2	-6.96±0.08	7700±1200	0.91

^aUncertainty in the parameter n was, on average, 0.007 and ranged from 0.001–0.02, for all data excluding site B of d(T₄G₄T₄G₄). For measures related to site B of d(T₄G₄T₄G₄), uncertainty in n was, on average, 0.04 and ranged from 0.01–0.09.

Table 4

Polyelectrolyte effect for DNA binding

DNA	T (°C)	$-\partial \ln K_A / \partial \ln [LiCl]$	Z	$\Delta G_{TM/LiCl}$ (kcal/mol)	$\Delta G_{electrostatic}$ (kcal/mol)	$\Delta G_{non-electrostatic}$ (kcal/mol)	$F_{non-electrostatic}$ (%)
TTGGGG	20	0.9±0.1	1.3±0.2	-7.4±0.2	-0.8±0.2	-7.6±0.2	90±2
TTTTGGGG	20	1.5±0.2	2.1±0.3	-7.7±0.2	-1.5±0.2	-8.1±0.2	84±2
GGGTTTGGGG	20	1.4±0.3	2.0±0.3	-7.8±0.2	-1.6±0.2	-8.2±0.2	84±2
GGGGTTTGGGG	20	1.8±0.1	2.5±0.1	-7.4±0.1	-1.5±0.2	-7.9±0.1	84±2
(A) TTTTGGGGTTTGGGG	25	1.4±0.2	2.0±0.3	-8.8±0.4	-1.6±0.5	-9.2±0.4	85±4
(B) TTTTGGGGTTTGGGG	25	0.6±0.1	0.9±0.1	-6.5±0.1	-0.5±0.1	-6.7±0.1	93±2

Table 5

Thermodynamic parameters for nucleotide substitution variants in 0.15 M LiCl

DNA	T (°C)	ΔH (kcal/mol)	ΔS [cal/(mol K)]	ΔG (kcal/mol)	K_D -DNA (nM)	n^d
GGGTTTGGGG	20	-5.06±0.02	16.1±0.1	-9.78±0.03	51±3	1.03
GGGTTTGGGG	25	-6.65±0.02	10.2±0.1	-9.66±0.04	80±5	1.08
GCGTTTGGGG	20	-4.01±0.04	17.8±0.3	-9.23±0.09	130±20	1.02
GCGTTTGGGG	25	-5.97±0.03	12.6±0.2	-9.73±0.05	72±6	1.01
GGGTTTGGCG	20	none detected	—	—	—	—
GGGTTTGGCG	30	none detected	—	—	—	—
(A) TTTTGGGGTTTGGGG	25	-1.58±0.04	30.8±0.6	-10.8±0.2	13±4	0.94
(B) TTTTGGGGTTTGGGG	25	-5.3±0.2	6.4±0.7	-7.18±0.06	5600±600	1.03
(B') TTTTGGGGTTTGGCG	25	-0.3±0.2	26±1	-8.0±0.3	1400±700	1.01
(A') TTTTGGGGTTTGGCG	25	-10±4	-12±10	-6.4±0.2	20,000±7000	1.2
TTTTGGGGTTTT	25	-8.61±0.04	-1.5±0.2	-8.15±0.3	1000±50	1.03

^dUncertainty in the parameter n was, on average, 0.008 and ranged from 0.002–0.03, for all data excluding “site B” and “site A” of d(TTTTGGGGTTTGGCG). For “site B” and “site A” of d(TTTTGGGGTTTGGCG) the uncertainty in n was 0.09 and 0.4 respectively.