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Impact of DNA Hairpin Folding Energetics on Antibody - ssDNA Association

Zhonghui Ou^{2,3}, Christopher A. Bottoms¹, Michael T. Henzl², and John J. Tanner^{1,2*}

¹*Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211, USA*

²*Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA*

Summary

Deposition of anti-DNA antibodies in the kidney contributes to the pathogenesis of the autoimmune disease, systemic lupus erythematosus. Antibodies that bind to hairpin-forming DNA ligands may be particularly prone to deposition. Here we report the first structure of a Fab complexed with hairpin-forming DNA. The ligand used for co-crystallization is 5'-d[CTG(CCTT)CAG]-3', which has a predicted hairpin structure consisting of a four-nucleotide loop (CCTT) and a stem of three base-pairs. The 1.95 Å resolution crystal structure of Fab DNA-1 complexed with this ligand shows that the conformation of the bound ligand differs radically from the predicted hairpin conformation. The three base-pairs in the stem are absent in the bound form. The protein binds to the last six nucleotides at the 3' end of the ligand. These nucleotides form a loop (TTCA) closed by a G:C base-pair in the bound state. Stacking of aromatic side chains against DNA bases is the dominant interaction in the complex. Interactions with the DNA backbone are conspicuously absent. Thermodynamics of binding are examined using isothermal titration calorimetry. The apparent dissociation constant is 4 micromolar, and binding is enthalpically favorable and entropically unfavorable. Increasing the number of base pairs in the DNA stem from 3 to 6 decreases binding affinity. These data suggest a conformational selection binding mechanism in which the Fab binds preferentially to the unstructured state of the ligand. In this interpretation, the ligand binding and ligand folding equilibria are coupled, with lower hairpin stability leading to greater effective binding affinity. Thus, preorganization of the DNA loop into the preferred binding conformation does not play a major role in complexation. Rather, it is argued that the stem of the hairpin serves to reduce the degrees of freedom in the free DNA ligand, thereby limiting the entropic cost attendant to complexation with the Fab.

Keywords

anti-DNA antibodies; X-ray crystallography; systemic lupus erythematosus; hairpin DNA; isothermal titration calorimetry; conformational selection

Introduction

Anti-DNA antibodies (Abs) are part of the autoimmune response associated with systemic lupus erythematosus (SLE).^{1–3} They play an important role in the pathology of the disease

*Corresponding author, E-mail address of the corresponding author: tannerjj@missouri.edu.

³Current address: Laboratory of Molecular Medicine, Children's Hospital/HHMI, 320 Longwood Avenue, Enders 673, Boston, MA 02115

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because deposition of anti-DNA antibodies in kidney tissue can induce renal disease. Three different deposition pathways have been observed.^{2, 4–11} First, anti-DNA Abs bind to DNA immobilized to the glomerular basement membrane. Second, anti-DNA Abs form complexes with nucleosomes released from apoptotic cells, and these complexes then deposit in the kidney. Third, anti-DNA Abs cross-react with non-DNA glomerular components by binding to molecules that mimic DNA. Because the binding of anti-DNA Abs to either DNA or ligands that mimic DNA is common to all three pathways, disruption of these interactions has been proposed as a novel drug design strategy.^{3, 8, 12–15} Thus, structural and thermodynamic analysis of Ab-DNA recognition is of interest.

Anti-DNA Abs that bind to hairpin-forming DNA ligands may be particularly important in the pathogenesis of SLE, based on seminal work from Glick's group on Ab 11F8.^{7, 16, 17} 11F8 was isolated from a panel of anti-DNA monoclonal Abs from an autoimmune MRL MpJ-lpr/lpr mouse. The pathogenicity of 11F8 has been demonstrated by mouse studies showing that 11F8 localizes to kidney tissue by binding to DNA adherent to the glomerular basement membrane, resulting in renal damage.⁷ *In vitro* evolution methods were used to identify tight binding DNA ligands for 11F8. These studies identified a 17-nucleotide DNA hairpin ligand (denoted LIG1-17) that binds to 11F8 with nanomolar affinity.¹⁸ Three stable hairpin conformations are predicted for LIG1-17 in solution (Figures 1a – 1c). The thermodynamics and kinetics of binding of 11F8 to LIG1-17 have been extensively studied.^{12, 17, 18} Based on this work, it has been proposed that the stem of the hairpin contributes to high affinity binding by preorganizing the loop into the preferred binding conformation.¹⁸ Although molecular modeling has been done for this system, crystallization of the 11F8/LIG1-17 complex has been unsuccessful.¹⁹

The focus of the present study is the recombinant anti-ssDNA antigen-binding fragment (Fab) DNA-1. DNA-1 was isolated from a combinatorial bacteriophage display library of IgG fragments derived from the immunoglobulin repertoire of an autoimmune SLE-like MRL/lpr mouse.²⁰ Like 11F8, DNA-1 shows a marked preference for binding thymine-rich ssDNA ligands over dsDNA.²¹ DNA-1 and 11F8 are also similar in terms of complementarity-determining region (CDR) loop sequences (Figure 2). There is only one amino acid difference in the light (L) chain CDRs, and the heavy (H) chain CDRs share 61 % identity.

Given the similarity between DNA-1 and 11F8, we investigated the binding of hairpin-forming DNA ligands to Fab DNA-1. Here we report the results of this investigation, including a 1.95 Å resolution crystal structure of DNA-1 complexed with a DNA ligand corresponding to nucleotides 5–14 of LIG1-17 (denoted LIG5-14, Figure 1d) and isothermal titration calorimetry (ITC) data for the binding of LIG1-17 and LIG5-14 to DNA-1.

Results

Description of the Overall Structure

The structure of Fab DNA-1 complexed with LIG5-14 was determined to 1.95 Å resolution (Table 1). The asymmetric unit consists of two Fabs (Fab 1 and 2), two LIG5-14 molecules, one PEG fragment and 400 water molecules (Figure 3, Table 1). The light/heavy chains are denoted L/H in Fab 1 and A/B in Fab 2. The residue numbering scheme and CDR definitions follow the standard Kabat conventions.^{22, 23} The two DNA strands have chain identifiers M and N. Strand N primarily interacts with Fab 1 while strand M primarily interacts with Fab 2 (Figure 3).

As expected, both Fab molecules display the well-characterized immunoglobulin fold.²⁴ Their overall conformations are quite similar, as indicated by root mean square differences (RMSDs) of 0.7 Å for main chain atoms and 1.0 Å for all atoms. Furthermore, the conformations of the

CDRs in Fab1 are similar to those in Fab 2. The RMSDs for the individual CDRs, after superimposition of the respective loops, are in the range 0.08 – 0.28 Å for main chain atoms and 0.15 – 1.1 Å for all atoms.

LIG5-14 is predicted to form a hairpin in solution with three Watson-Crick base pairs (C5:G14, T6:A13, G7:C12) in the stem and four nucleotides (C8, C9, T10, T11) in the loop (Figure 1d). The bound DNA molecule, however, has a strikingly different conformation (Figure 1e, Figure 3). The bound ligand consists of a 4-nucleotide loop formed by T10, T11, C12 and A13, a base pair (C9:G14), and a 5'-terminal tail of 4 nucleotides (C5, T6, G7, C8). Note that nucleotides C12 and A13, which appear in the loop of the bound ligand, are part of the stem of the consensus hairpin (compare Figures 1d and 1e). And the base-paired nucleotides of the bound ligand, C9 and G14, reside in the loop and stem, respectively, of the predicted hairpin.

The two DNA strands interact through intermolecular Watson-Crick hydrogen bonding involving G7 and C8 (Figure 3, inset). This interaction is presumably a consequence of crystal packing. As a result of this base pairing, the two strands form a structure reminiscent of a DNA dumbbell (Figure 3).

Protein-DNA Interactions

Each Fab interacts with nucleotides 9–14 of one DNA strand and nucleotides 5–6 of the other strand. Because the protein-DNA interactions formed by the two Fabs are nearly identical, just one set of interactions will be described.

The major protein-DNA interface involves nucleotides 9–14, which form the loop and base-pair of the bound ligand. The DNA loop is oriented so that the bases interact with the Fab, while the phosphoribose backbone is directed away from the protein (Figure 4). The surface area buried between the Fab and nucleotides 9–14 is 1115 Å², indicating extensive protein-DNA interaction. Each Fab in the asymmetric unit also interacts with nucleotides 5 and 6 of the DNA strand bound by the other Fab (Figure 3). The latter interface buries only 416 Å². Based on these surface area values, we suggest 1) that the protein-DNA interactions involving nucleotides 9–14 represent those formed in solution and 2) that those involving nucleotides 5–6 are artifacts of crystal packing. Moreover, as discussed in the next section, the interactions with the two thymine bases of the DNA loop (T10, T11) are identical to those observed in our previous structures of DNA-1 complexed with dT₃ and dT₅, which also suggests that the protein interactions with nucleotides 9–14 reflect solution behavior. We now describe the binding of the DNA loop to Fab DNA-1.

All three L chain CDRs and HCDR3 are involved in binding nucleotides 9–14 (Figure 4). The DNA loop wraps around the tip of HCDR3 such that the side chains of HCDR3 residues Arg H98, Tyr H100 and Tyr H100A contact the inside surface of the DNA loop (Figure 4). This configuration allows Arg H98 and Tyr H100 to pack on top of the C9:G14 base-pair (Figure 4). The plane of the Arg H98 guanidinium is nearly parallel with the base of G14, and Arg H98 does not form electrostatic interactions with the DNA backbone (Figure 5). Planar packing of Arg H98 with G14 presumably results in favorable cation- π interaction. Note also that the C9:G14 base-pair has a perceptible twist, which might be due steric interactions between Tyr H100 and the base of C9 (Figure 5).

Recognition of bases T10 and T11 seems to be central for binding, based on the extent of interaction with the Fab. The bases of T10 and T11 insert edgewise into a narrow groove lined by the tyrosine side chains of L32, L49, H100, and H100A (Figure 4). T10 is sandwiched between the phenol groups of L32 and H100, while T11 is sandwiched between the phenol groups of L49 and H100A (Figure 4, Figure 5). T10 also engages in significant hydrogen bonding (Figure 5). The carbonyls of T10 accept hydrogen bonds from the side chain of His

L91 and the backbone amine of Gly L93, which both belong to LCDR3. Also, the base of T10 donates a hydrogen bond to the main chain carbonyl of His L91. Thus, the hydrogen bond capacity of T10 is fully utilized in binding. T11 also forms hydrogen bonds, but to a lesser extent than T10. The O4 carbonyl of T11 accepts a hydrogen bond from the amine of Ala H100B of HCDR3, and the N3 of T11 forms a water-mediate hydrogen bond with the carbonyl of Ala H100B (Figure 5).

Nucleotides C12 and A13 form fewer interactions than T10 and T11. These two bases form an aromatic stack with Tyr H97 of HCDR3, such that A13 is sandwiched between C12 and Tyr H97 (Figure 4, Figure 5).

All of the aforementioned interactions involve DNA bases. We note that interaction with DNA bases (aromatic stacking, hydrogen bonding) is the dominant recognition theme for anti-DNA Abs, as evidenced by previous structural work on BV04-01,²⁵ ED-10²⁶ and DNA-1.^{27, 28} Indeed, there is only one interaction with the LIG5-14 backbone, which is a hydrogen bond between Asn L50 of LCDR2 and the phosphate group of T11 (Figure 5). The interaction distance is 3.6 Å in Fab 1 and 3.4 Å in Fab 2, which is rather long for a hydrogen bond, suggesting that the interaction is not particularly strong.

Comparison to other Fab/DNA crystal structures

Prior to this work, four crystal structures had been determined for DNA-ligated anti-DNA Fabs: Fab BV04-01 complexed with d(pT)₃,²⁵ DNA-1 complexed with dT₃²⁷ and dT₅,²⁸ and most recently, Fab ED-10 complexed with 5'-TCAACC-3'.²⁶ The DNA-1/LIG5-14 structure is unique in that the bound ligand forms a closed loop featuring a base-pair, whereas the ligands in the other structures adopt linear conformations.

CDR usage differs among the Fabs crystallized to date. HCDR1 and HCDR2 do not participate in binding nucleotides 9–14 in DNA-1; however, these CDRs form important contacts in the BV04-01 and ED-10 complexes. On the other hand, LCDR2 forms contacts in all the DNA-1 structures, yet this is the only CDR not involved in binding in BV04-01 and ED-10.

Comparison of the present structure to the DNA-1/dT_n structures shows that the interactions described here for T10 and T11 are nearly identical to those in the DNA-1/dT_n structures (Figure 6). In both cases, the 5' thymine base stacks between Tyr L32 and Tyr H100, while the 3' thymine is sandwiched between the tyrosine side chains of L49 and H100A. Moreover, the hydrogen bond interactions are remarkably similar. In both structures, the 5' thymine forms three hydrogen bonds with LCDR3 residues His L91 and Gly L93, while the 3' thymine forms a hydrogen bond with Ala H100B.

The binding site for T10 corresponds to the "ssDNA-antibody recognition module" or "D-ARM", which we proposed to be a conserved binding motif used by anti-ssDNA Abs.²⁸ The motif consists of aromatic stacking interactions from LCDR1 and HCDR3, and hydrogen bonding interactions from LCDR3. As discussed by Sanguineti, *et al.*, this motif is utilized by ED-10 to bind a cytosine ring.²⁶ Thus, all Fab/DNA crystal structures determined to date feature this binding motif.

We previously determined two structures of ligand-free DNA-1²⁷ (PDB entries 1XF3, 1XF4). The ligand-free DNA-1 structures revealed that HCDR3 is disordered in the absence of a bound ligand and that this critical CDR is ordered when bound to dT_n.²⁷ HCDR3 is likewise ordered when bound to LIG5-14, but the degree of ordering appears to be more extensive than in the dT_n complexes. In particular, the side chains of HCDR3 residues Tyr H97 and Arg H98 are disordered in the two dT_n complexes, although the backbone is ordered. In the DNA-1/LIG5-14 complex, however, electron density for Arg H98 is quite strong, presumably due to the stacking

interaction with the base of G14 and the water mediated hydrogen bond with C9. Density for the side chain of Tyr H97 was also sufficient to include this residue in the model at full occupancy, although the density feature was not as strong as that for Arg H98. Stabilization of Tyr H97 is likely due to the stacking interaction with A13 (Figure 5).

Binding Studies

Isothermal titration calorimetry (ITC) was used to examine the binding of LIG1-17 and LIG5-14 to DNA-1. Results of these studies are shown in Figure 7 and Table 2. For comparison, thermodynamic parameters determined for dT₅ using ITC are also included in Table 2. We note that the solution conditions used here are identical to those used to study binding of dT₅, that is, 0.01 M imidazole, 0.1 M NaCl, pH 7.0, 298 K.

Under these conditions, DNA-1 exhibits marginally higher affinity for the smaller of the two hairpin ligands, LIG5-14. The association constant for LIG5-14 is $K_A = 2.5 \times 10^5 \text{ M}^{-1}$, which corresponds to $K_d = 4.0 \text{ } \mu\text{M}$ and $\Delta G = -7.3 \text{ kcal/mol}$. The values for LIG1-17 are $K_A = 1.4 \times 10^5 \text{ M}^{-1}$, $K_d = 7.1 \text{ } \mu\text{M}$ and $\Delta G = -7.0 \text{ kcal/mol}$. Both binding events are highly exothermic (Figure 7), with ΔH values of -15.1 kcal/mol for LIG5-14 and -13.2 kcal/mol for LIG1-17. Thus, the difference in affinity for the two ligands reflects a more favorable enthalpy of association for LIG5-14 coupled with a higher entropic penalty (Table 2).

The association constants for the two hairpin ligands are 2–4 times larger than that of dT₅ (Table 2). This difference reflects lower entropic penalties of binding for the hairpin ligands coupled with less favorable enthalpy changes.

Discussion

Impact of DNA hairpin folding energetics on antibody - ssDNA association

Anti-DNA Abs are widely studied because of their role in the pathology of SLE. Compounds that bind anti-DNA Abs, thereby preventing their deposition in the kidney, have potential as SLE drugs.^{8, 12} Glick and co-workers have shown that Fab 11F8 exhibits a marked preference for single-stranded sequences having the potential to adopt hairpin conformations. The pronounced similarities between 11F8 and DNA-1 (see below) stimulated efforts to obtain high-resolution structural data on DNA-1 complexed with a hairpin ligand. Although we have been unable to obtain crystals of the DNA-1/LIG1-17 complex, DNA-1 readily co-crystallizes with the abbreviated hairpin sequence, LIG5-14.

Unexpectedly, we find that the conformation of bound LIG5-14 differs radically from the predicted hairpin conformation (compare Figures 1d and 1e). The three base-pairs in the stem are absent in the bound form, replaced by a single C9:G14 base pair. Moreover, the major recognition locus, consisting of T10 and T11, resides in the 5' half of the loop in the bound form (Figure 1e) rather than the 3' half, as in the hairpin (Figure 1d).

It should be emphasized that the hairpin conformation of LIG5-14 is only marginally stable at 25 °C. Theoretical thermodynamic stability data for the hairpin conformations of LIG5-14 and LIG1-17 are listed in Table 3. The free energy of folding for LIG5-14 is just -0.2 kcal/mol . Assuming the existence of two states and Boltzmann statistics, the hairpin and unstructured states account for 58 % and 42% of the ligand population, respectively. Because the bound DNA conformation does not resemble the hairpin conformation, and because the unfolded state is significantly populated, we propose that DNA-1 binds preferentially to the unstructured state of LIG5-14. The similarity of the DNA-1/LIG5-14 structure to those of DNA-1 complexed with dT₃ and dT₅ lends further support to this argument.

Despite our inability to obtain crystals of DNA-1 complexed with LIG1-17, we believe that the dominant binding determinants described here – such as the protein interactions with T10 and T11, and formation of the C9:G14 base-pair – are likewise valid for LIG1-17. This assertion is based on three observations. First, the predicted hairpin LIG5-14 (Figure 1d) is identical to that formed by the loop and proximal stem in the predicted LIG1-17 hairpins (Figures 1a–1c). Additionally, the two ligands are bound with similar affinities (Table 2). Finally, both binding events are strongly enthalpy driven with an unfavorable entropy change (Table 2).

The potential hairpin conformations of LIG1-17 (Figure 1a–1c) are somewhat more stable than the LIG5-14 hairpin, with predicted folding free energies of -1.3 , -1.0 , and -0.6 kcal/mol, respectively (Table 3). Nevertheless, the unfolded state is significantly populated (5 %) at 25 °C. Thus, it is likely, as for LIG5-14, that DNA-1 preferentially binds to the unstructured form of LIG1-17.

Glick has proposed for 11F8 that the hairpin stem serves to preorganize the DNA loop into the preferred binding conformation.¹⁸ In fact, the structural and energetic data presented here suggest that the stem plays a somewhat more prosaic role. Specifically, it serves to reduce the degrees of freedom in the free DNA ligand, thereby limiting the entropic cost attendant to complexation with the Fab. Our model for the Fab-hairpin interaction is represented by Scheme 1 for the limiting case of a single hairpin conformation and two-state hairpin folding.

In Scheme 1, L_F and L_U are the folded and unfolded conformations of the ligand, respectively; P is the protein; and PL is the protein-ligand complex. K_1 is the equilibrium constant for ligand folding. K_2 is the association constant for the Fab binding to the unfolded ligand.

The observed, or effective, equilibrium binding constant, K_{eff} , can be expressed as follows: $K_{eff} = K_2/(1 + K_1)$. Similar ligands, such as LIG1-17 and LIG5-14, might be expected to have similar values of K_2 . In this case, differences in K_{eff} primarily reflect differences in ligand-folding thermodynamics, with lower hairpin stability leading to greater effective binding affinity. Indeed, we observe such behavior for LIG1-17 and LIG5-14. DNA-1 exhibits higher affinity for LIG5-14, whose hairpin conformation has lower stability than any of the three hairpin conformations of LIG1-17.

The binding enthalpies and entropies for LIG1-17 and LIG5-14 also support the idea that binding is coupled to hairpin unfolding. LIG1-17 binding is less exothermic ($\Delta\Delta H = +1.9$ kcal/mol) than LIG5-14 binding. Because the LIG1-17 hairpins enjoy greater enthalpic stabilization than the LIG5-14 hairpin, LIG1-17 unfolding offsets a larger portion of the intrinsically favorable binding enthalpy.

Conversely, LIG1-17 binding is entropically more favorable ($-T\Delta\Delta S = -1.6$ kcal/mol) than LIG5-14 binding. Due to their intrinsically higher stabilities, unfolding of the LIG1-17 hairpins yields a greater entropic reward. In other words, the more favorable entropy of LIG1-17 hairpin unfolding offsets a larger portion of the intrinsic entropy penalty of binding.

Although dT₅ is less structured than either LIG1-17 or LIG5-14, it displays lower affinity for DNA-1. All else being equal, this result would seem to conflict with the coupled unfolding-binding model. However, LIG5-14, and presumably LIG1-17, can enjoy additional interactions in the bound state that are denied to dT₅. These interactions include the C9:G14 base pair, stacking of Arg H98 with G14, van der Waals interactions between Tyr H100 and C9, and stacking of Tyr H97 against A13.

Despite this inherent difference between the hairpin ligands and dT_n, DNA-1 binding is enthalpically favorable and entropically unfavorable for all three ligands. The enthalpy change is significant, ranging from -13.2 kcal/mol for LIG1-17 to -16.8 kcal/mol for dT₅. As described above and previously by Schuermann and coworkers,²⁷ HCDR3 is disordered in the ligand-free state and becomes ordered upon ligand binding. Such folding of the protein upon association likely contributes substantially to the negative binding enthalpy changes observed for DNA-1.

The DNA-1/hairpin system appears to be an example of "induced-fit by conformational selection", a term coined by Leder *et al.* in 1995 to describe anti-peptide antibodies that seemed to induce helical structure in random coil peptides.²⁹ In conformational selection, the protein selects those ligands having conformations suitable for binding. It contrasts classical induced-fit binding, in which ligand binding begins with the formation of a low affinity encounter complex, and conformational adjustments in the ligand and/or receptor occur to achieve the final high affinity complex.³⁰ The salient difference between the two is that induced-fit posits conformational changes occurring during binding, whereas conformational selection sees conformational change as part of the dynamic equilibrium of the free ligand. Similar to the peptide ligands that bind antibodies via conformational selection,^{31, 32} the ssDNA ligands studied here are highly flexible and exhibit folding/unfolding equilibria. It is hard to imagine the substantial rearrangement of the DNA hairpin into the ligand-bound conformation occurring in the confines of an encounter complex. Moreover, the non-hairpin populations are substantial for LIG5-14 and LIG1-17. It thus seems more plausible that DNA-1 selects a ligand with the appropriate conformation from the unfolded population.

Relevance to 11F8

11F8 is a pathogenic anti-DNA Ab that binds with high affinity ($K_d = 10$ nM) and specificity to LIG1-17. However, efforts to crystallize 11F8 have thus far proven unsuccessful.¹⁹ A desire to provide structural data relevant to Ab 11F8 was a major motivation for this study. We note that the theoretical models of 11F8 complexed with DNA^{12, 17, 19} are based, in part, on the DNA-1/dT_n crystal structures.

Similar to DNA-1, binding of LIG1-17 and poly(T) DNA ligands to 11F8 is enthalpy driven with an unfavorable entropy change (based on van't Hoff analysis of fluorescence quenching data). Thus, the DNA-1/LIG5-14 structure might be relevant for understanding DNA recognition by 11F8. It is possible, if not likely, that the Fab interactions with T10 and T11 observed here apply also to 11F8. For example, the DNA-1/LIG5-14 structure is consistent with chemical protection data for 11F8-complexed LIG1-17 showing that T10 and T11 are the most highly protected bases.¹⁶⁻¹⁸ Indeed, these bases are the most highly buried of all the bases in our structure. Moreover, all the residues that contact T10 and T11 in our structure are present in the sequence of 11F8: Tyr L32, Tyr L49, His L91, Tyr H100, and Tyr H100A (DNA-1 residue numbering, see Figure 2). Based on these observations, and considering the discussion in the previous section, we suggest that 11F8, like DNA-1, preferentially binds to relatively unstructured conformations of DNA hairpins.

The coupled unfolding-binding model proposed here is supported by binding measurements in the Glick laboratory on the 16-nucleotide ligand corresponding to deletion of G4 from LIG1-17 (Δ dG4). A single hairpin conformation is predicted for Δ dG4, corresponding to the conformation shown in Figure 1b without the G4 bulge. The predicted folding free energy of this hairpin -5.2 kcal/mol, as compared to values of about -1 kcal/mol for the hairpin conformations of LIG1-17. The coupled equilibria argument would predict a lower affinity for Δ dG4 than LIG1-17, due to the higher stability of the former ligand. In fact, the dissociation constant for binding of Δ dG4 to 11F8 is 90 nM, nine times larger than that for LIG1-17 ($\Delta\Delta G = +1.3$ kcal/mol).¹⁸ Thus, the G4 bulge may increase the affinity of LIG1-17 for 11F8

by destabilizing the hairpin and, thereby, increasing accessibility to less structured ligand conformations.

Although DNA-1 and 11F8 share a common thermodynamic signature of binding, the association constant for binding of LIG1-17 is 700 times larger for 11F8. Thus, it is likely that 11F8 employs additional binding determinants not seen in our structure. Notable sequence differences (see Figure 2) include additional Arg residues in HCDR1 and HCDR3 of 11F8 (Arg31, Arg96). These residues are Ser31 and Gly 96 in DNA-1.

Bobeck and coworkers have shown that these additional Arg residues are important for high affinity binding of LIG1-17 to 11F8. Mutation of Arg96 to Lys causes a 60-fold decrease in affinity ($K_d = 590$ nM, $\Delta G = -8.5$ kcal/mol),¹⁷ whereas the Arg31Ser mutation decreases affinity by a factor of 30 ($K_d = 286$ nM, $\Delta G = -8.9$ kcal/mol).³³ Assuming simple additivity of free energy changes, these two residues alone nearly account for the difference of affinities of 11F8 and DNA-1 for LIG1-17. It is difficult to infer a role for Arg96 from the DNA-1/LIG5-14 structure because HCDR3 of 11F8 is one residue shorter than that of DNA-1. On the other hand, *in silico* mutagenesis of DNA-1/LIG5-14 suggests that Arg31 could interact with the bases of A13 and C12.

Bobeck and coworkers have argued that somatic mutation of Ser31 to Arg is critical in the transition to pathogenicity because this change shifts the binding site toward the heavy chain.³³ As we see no ligand contacts from HCDR1 or HCDR2 in DNA-1 structures, it is likely that Arg31 is a critical difference between 11F8 and DNA-1. The specific structural roles that these extra arginine residues play in ssDNA recognition remain to be seen.

Materials and methods

Crystallization and X-ray diffraction data collection

Expression and purification of the recombinant Fab DNA-1 were performed as previously described.³⁴ The purified protein was dialyzed into 10 mM Tris, 50 mM NaCl, pH 7.0 and concentrated to 16 mg/ml using a stirred Amicon ultrafiltration cell (30 kDa MW cutoff). Protein concentration was determined spectrophotometrically using a theoretically determined optical density value of $1.6 \text{ mg}^{-1} \text{ cm}^{-1} \text{ ml}$ ($\lambda = 280 \text{ nm}$).³⁵

The DNA oligonucleotide LIG5-14 (5'-CpTpGpCpCpTpTpCpApG-3') was purchased from Integrated DNA Technologies, Inc. (standard desalting preparation) and used without further purification. Prior to crystallization, LIG5-14 was dissolved in 10 mM Tris, 50 mM NaCl, pH 7.5 to achieve a 4.7 mM stock solution. The DNA-1 and LIG5-14 stock solutions were mixed so that the molar ratio of ligand to protein was in the range $[\text{LIG5-14}]/[\text{DNA-1}] = 3 - 5$.

Crystallization experiments were performed in hanging drops by mixing 3 μl of the DNA-1/LIG5-14 solution with 3 μl of the reservoir at 295 K. Screening trials using commercially available crystal screen kits produced initial crystals grown in Wizard I formulations 10 and 19. These crystals were in the shape of rectangular solids. After several rounds of optimization, reservoir solutions containing 0.1 M Tris pH 7.0 and 12–26% (w/v) PEG 2000 MME were found to yield the best crystals. The crystals have space group $P2_12_12_1$ with unit cell dimensions $a = 84.8 \text{ \AA}$, $b = 90.4 \text{ \AA}$, $c = 128.1 \text{ \AA}$. The asymmetric unit contains two Fab molecules and two LIG5-14 DNA molecules, with solvent content of 51% and $V_M = 2.5 \text{ \AA}^3 \text{ Da}^{-1}$.³⁶

Several crystals were prepared for cryogenic X-ray data collection by replacing the mother liquor with 0.1 M Tris pH 7.0, 25% (w/v) PEG 2000 MME and 10% (v/v) PEG 200. The crystals were then picked up with Hampton mounting loops and plunged into liquid nitrogen.

Three X-ray diffraction data sets were collected at Advanced Photon Source beamline 19-ID. The 1.95 Å resolution data set used for refinement consisted of a 100° scan with oscillation width of 0.5° per frame, exposure time of 10 s per frame, detector distance of 160 mm, and detector 2θ angle of zero. Two data sets were collected at low energy ($\lambda = 1.740$) for use in anomalous difference Fourier analysis. Low energy data set 1 consisted of a 171° scan with oscillation width 0.5°, exposure time of 5 s per frame, detector distance of 120 mm and detector angle of -20°. Low energy data set 2 consisted of a 219° scan collected with oscillation width 0.5°, exposure time of 10 s per frame, detector distance of 120 mm and detector angle of -10°. All three data sets were integrated and scaled with HKL2000.³⁷ See Table 1 for data collection and processing statistics.

Model building, refinement, and analysis

Molecular replacement calculations were performed with MOLREP³⁸ using conserved and variable superdomain search models obtained from the DNA-1/dT₅ structure (PDB entry 1I8M). The best solution, which had correlation coefficient of 0.3, was obtained for 2 Fabs per asymmetric unit in space group P2₁2₁2₁.

Initially, O³⁹ was used for model building and CNS⁴⁰ was used for structure refinement. Simulated annealing was used in the first round of refinement, and conjugate gradient refinement was used thereafter. Non-crystallographic symmetry restraints were not used. Cross validation was used with a randomly chosen test set of 3596 reflections and a working set of 63,514 reflections. After the protein model was nearly complete, 54 water molecules were added, using the water picking algorithm of CNS. At this stage, the residual values were $R_{cryst} = 0.297$ and $R_{free} = 0.336$, and the $2F_o - F_c$ and $F_o - F_c$ maps clearly showed the presence of bases bound to the CDRs, as well as bases engaged in Watson-Crick hydrogen bonding. In particular, there was strong density indicating that two bases were inserted edgewise into a groove lined by the tyrosine side chains of L32 and L49 on one side and H100 and H100A on the other side. These features were interpreted as representing thymine bases T10 and T11 and formed the starting point for modeling the DNA.

COOT⁴¹ was used to model DNA and for all subsequent model building sessions. Modeling of DNA was guided by anomalous difference Fourier maps calculated from the model phases and anomalous differences from the low energy data sets. These maps were calculated to a resolution of 3.0 Å from each of the two low energy data sets and then averaged together using CNS. It was found that the averaged map was more informative than either of the individual maps for locating P atoms (Figure 3, inset).

Models built with COOT were refined with REFMAC5, including TLS refinement.⁴² For the protein, the following groups were used for TLS refinement: A1 – A108, A109 – A213, L1 – L108, L109 – L213, B1 – B113, B114 – B213, H1 – H113, and H114 – H213. Hydrogen atoms were added in the riding positions in the refinement. After modeling of the DNA ligand was finished, additional water molecules and one PEG fragment were incorporated into the model. The two DNA strands were included as two separate groups in the final stage of the TLS refinement. The final model had residual values of $R_{cryst} = 0.207$ and $R_{free} = 0.256$. See Table 1 for refinement statistics. Analysis of protein backbone geometry with RAMPAGE revealed only one residue (Ser A77) outside of the favored and allowed regions of the Ramachandran plot. This residue appears in a loop, and its (ϕ , ψ) value of (62°, 94°) is on the edge of the allowed region. We note that this loop is far from the binding site.

Structure analysis was performed with CNS, COOT, CCP4,⁴³ and PyMOL.⁴⁴ Simulated annealing omit maps were calculated using PHENIX.⁴⁵

Theoretical predictions of DNA secondary structure and thermodynamics of hairpin folding were performed using the mfold server version 3.2 (<http://www.bioinfo.rpi.edu/applications/mfold/>).⁴⁶ Conditions of $[Na^+] = 100$ mM and $T = 25$ °C were used for these calculations.

Isothermal Titration Calorimetry

Binding studies were performed with a MicroCal VP-ITC at 298 K. The two oligonucleotides LIG5-14 and LIG1-17 (5'-CpGpAp GpCpTp GpCpCp TpTpCp ApGpTp CpG -3') were purchased from Integrated DNA Technologies, Inc. (standard desalting preparation) and used without further purification. Prior to ITC experiments, solutions of the DNA ligand and Fab were dialyzed separately into 0.01 M imidazole, 0.1 M NaCl, pH 7.0 buffer. For both titrations, Fab DNA-1 (0.03 mM) was placed in the calorimeter cell, and the oligonucleotide (0.4 mM) was added to the cell via the rotating stirrer-syringe. Blank titrations were performed by injecting the ligand (0.4 mM) into the buffer devoid of protein to correct for the heat of mixing and ligand dilution. Values of the association constant, enthalpy and entropy were obtained by fitting the integrated heats of association to a one-site binding model, using the software supplied with the instrument.

PDB accession code

Atomic coordinates and structure factor amplitudes have been deposited in the PDB⁴⁷ as entry 2FR4.

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Abbreviations used

Ab, antibody
 Fab, antigen-binding fragment

CDR, complementarity-determining region
SLE, systemic lupus erythematosus
PDB, protein data bank
V, variable
C, conserved
H, heavy
L, light
RMSD, root mean square difference
ITC, isothermal titration calorimetry
LIG1-17, ssDNA ligand 5'-d[CGAGCTG(CCTT)CAGTCG]-3'
LIG5-14, ssDNA ligand 5'-d[CTG(CCTT)CAG]-3'

Figure 1.

Secondary structure diagrams for the DNA ligands used in this study. The three predicted hairpin conformations for LIG1-17 are shown in (a) – (c). The predicted hairpin conformation of LIG5-14 is shown in (d). The conformation of LIG5-14 bound to Fab DNA-1 is depicted in (e). The black and gray circles denote G:C and T:A base-pairs, respectively. The open circle represents a G:T wobble base pair.

Figure 2.

Sequence alignment of the CDR regions of DNA-1 and 11F8. The sequence numbers above the alignment correspond to DNA-1. The CDRs of DNA-1 are as follows: LCDR1, 24–34; LCDR2, 50–56; LCDR3, 89–97; HCDR1, 31–35; HCDR2, 50–65; HCDR3, 95–102. The alignment was performed using the GeneStream web server (xylia.igh.cnrs.fr).

Figure 3.

Ribbon drawing of the asymmetric unit. The light and heavy chains are colored pink and blue, respectively. DNA strands M and N are colored yellow and green, respectively. Fab 1 (chains L and H) is shown in the lower right, while Fab 2 (chains A and B) is shown in the upper left. The black oval indicates the non-crystallographic two-fold axis, which relates the two Fab/DNA complexes. The inset shows the two DNA ligands with electron density maps. The orientation is identical to that of the main figure. The cyan map is a simulated annealing σ_A -weighted $F_o - F_c$ omit map contoured at 1.5σ . Prior to map calculation, the two DNA strands were deleted from the final model and input to slow cooling simulated annealing refinement in PHENIX (starting temperature = 5000 K). The magenta map is an averaged anomalous difference Fourier map calculated from the low energy data sets (2σ). Nucleotide numbers of strand M (yellow) are indicated.

Figure 4.

Overall view of the binding site for nucleotides 9–14, highlighting CDR usage. The CDRs are colored coded as follows: LCDR1, red; LCDR2, green; LCDR3, magenta; HCDR1 yellow; HCDR2, blue; and HCDR3, cyan.

Figure 5.

Stereo view of one of the Fabs interacting with nucleotides 9–14. Dotted lines indicated hydrogen bonds. DNA is colored green. Light chain residues are colored magenta and appear on the right hand side of the figure. Heavy chain residues are colored cyan and appear on the left hand side of the figure. Compared to Figure 4, the orientation is rotated by about 180° around the vertical axis.

Figure 6. Superposition of the DNA-1/LIG5-14 and DNA-1/dT₅ structures, highlighting the similarity of the environments of the thymine bases. DNA-1/LIG5-14 is colored green (DNA), pink (light chain) and cyan (heavy chain). DNA-1/dT₅ is colored silver. The black and red dotted lines denote hydrogen bonds in DNA-1/LIG5-14 and DNA-1/dT₅, respectively.

Figure 7.

ITC analysis of LIG5-14 and LIG1-17 binding to Fab DNA-1. The upper panel of each figure shows the raw calorimetric data, and the lower panel shows the corresponding integrated injection heats, corrected for the heat of dilution. The curves in the lower halves of each figure represent the best least squares fit to a one-site binding model. Prior to titration, LIG5-14, LIG1-17, and DNA-1 were dialyzed into 0.01 M imidazole, 0.1 M NaCl, pH 7.0. (a) ITC analysis of LIG5-14 binding to Fab DNA-1. The apparent thermodynamic parameters from the best-fit curve are $K_A = 2.5 \times 10^5 \pm 0.2 \times 10^5 \text{ M}^{-1}$, $\Delta H = -15.1 \pm 0.3 \text{ kcal/mol}$. (b) ITC analysis of LIG1-17 binding to Fab DNA-1. The apparent thermodynamic parameters from the best-fit curve are $K_A = 1.4 \times 10^5 \pm 0.1 \times 10^5 \text{ M}^{-1}$, $\Delta H = -13.2 \pm 0.5 \text{ kcal/mol}$.

Table 1Data Collection and Refinement Statistics^a

Data Collection	High energy	Low energy 1	Low energy 2
Wavelength (Å)	0.9786	1.7400	1.7400
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å)	a = 84.8 b = 90.4 c = 128.1	a = 85.1 b = 90.9 c = 128.3	a = 85.0 b = 91.1 c = 128.3
Resolution limits (Å)	50 - 1.95 (2.02 - 1.95)	50 - 2.45 (2.54 - 2.45)	50 - 2.45 (2.54 - 2.45)
Observations	285148	239174	254697
Unique reflections	70761	36631	37300
Completeness (%)	97.4 (97.1)	98.1 (91.9)	99.9 (99.9)
Mean I/σ (I)	23.8 (2.8)	25.3 (10.1)	28.1 (4.5)
R _{merge}	0.052 (0.420)	0.061 (0.170)	0.063 (0.434)
Refinement			
Number of used reflections	67110		
Protein atoms	6479		
DNA atoms	396		
Water molecules	400		
PEG fragments	1		
R _{cryst}	0.207 (0.267)		
R _{free}	0.256 (0.297)		
RMSD ^c			
Bond lengths (Å)	0.013		
Bond angles (°)	1.54		
Ramachandran plot ^d			
Favored (no. of residues)	820		
Allowed (no. of residues)	20		
Outliers (no. of residues)	1		
Average B-factors (Å ²)			
Protein	40		
DNA	37		
Water	41		
PEG	58		
PDB entry	2FR4		

^aValues for the outer resolution shell of data are given in parenthesis.

^b5% R_{free} test set.

^cCompared to the Engh and Huber parameters.⁴⁸

^dThe Ramachandran plot was generated with RAMPAGE.⁴⁹

Table 2

Isothermal titration calorimetry data for ligand binding to Fab DNA-1 at 298K

Ligand	K_A (M^{-1})	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
LIG5-14	2.5×10^5	-15.1	7.8
LIG1-17	1.4×10^5	-13.2	6.2
dT ₅ ^a	0.6×10^5	-16.8	10.3

^aData from Schuermann *et al.*⁵⁰

Table 3Calculated thermodynamic stability data for hairpin conformations of LIG1-17 and LIG5-14^a

Ligand	ΔG_{fold} (kcal/mol)	ΔH_{fold} (kcal/mol)	$-T\Delta S_{\text{fold}}$ (kcal/mol)
LIG5-14	-0.2	-19.6	19.4
LIG1-17 conformation a	-1.3	-29.1	27.8
LIG1-17 conformation b	-1.0	-46.8	45.8
LIG1-17 conformation c	-0.6	-22.1	21.5

^a Calculations were performed using the mfold server assuming T = 25°C and [Na⁺] = 100 mM.⁵⁰