C h a p t e r 3

AFFINITY, STOCHIOMETRY AND CONFORMATION OF SUBSTRATE DNA BOUND TO Zα

Introduction

Zα is a 63-residue domain with a (α+β) helix-turn-helix (α+βHTH) fold, showing structural similarity to other members of the widespread family of α+βHTH B-DNA binding protein domains [5]. It has been shown by several biochemical and spectroscopic studies *in vitro* [3,15,169], and by the crystal structure of Zα bound to substrate DNA [6] that the DNA complexed with Zα adopts the left-handed Z-conformation. Previous studies [3] utilized polyd(CG) to determine the affinity of Zα to Z-DNA. For the investigation of the Za/Z-DNA interaction by high resolution NMR spectroscopy, the size of the Z-DNA substrate was reduced to obtain a minimal molecular weight for the Za/Z-DNA complex. Thus, a defined hairpin substrate, $d(CG)_3T_4(CG)_3$, was used to determine the binding constant and stochiometry of Zα bound to Z-DNA in solution. By analytical ultracentrifugation and CD spectroscopy, it was found that two Zα domains bind to one hairpin in the Z-DNA conformation, while free Zα exists as a monomer. The equilibrium dissociation constant (K_d) is approximately 30 nM, as measured by both analytical ultracentrifugation and surface plasmon resonance (SPR).

A C125S mutant of Zα which lacks cysteines that can form disulfide bridges, was better suited for investigation by high resolution NMR spectroscopy than wild-type. Therefore, this mutant which showed a distinct binding behavior in previous bandshift experiments [15], was studied by the same techniques as wild-type, yielding a virtually identical K_d and stochiometry. Taken together, this work demonstrates that the formation of a compact ternary $(Z\alpha)_2$/Z-DNA complex requires a binding site of no more than six basepairs. In contrast, B-DNA binding (α+β)HTH proteins usually require a recognition site of 8 or more basepairs.

Results and Discussion

In this study the stochiometry and affinity of the Zα domain to a $d(CG)_3T_4(CG)_3$ hairpin, which contains six bp of alternating d(CG) substrate DNA, was determined. The two $d(CG)_3$ strands of this hairpin were tethered through a T_4 loop, which increases the melting temperature by 40°C ensuring that a stable duplex stem is present at room temperature [170]. Analytical ultracentrifugation verified that $d(CG)_3T_4(CG)_3$ shows the molecular weight of a hairpin (measured: 4850, theoretical: 4864) rather than that of higher molecular weight duplexes with T_4 bulges. $d(CG)_3T_4(CG)_3$, both alone and in complex with Zα, exhibited peaks between 12.9 and 13.2 ppm in 1D ^{1}H NMR experiments in H_2O at 4°C [data not shown]. Such peaks are characteristic of hydrogen bonded guanosine imino protons in duplex DNA. These data indicate that $d(CG)_3T_4(CG)_3$ exists in solution as a double-stranded $d(CG)_3$ stem

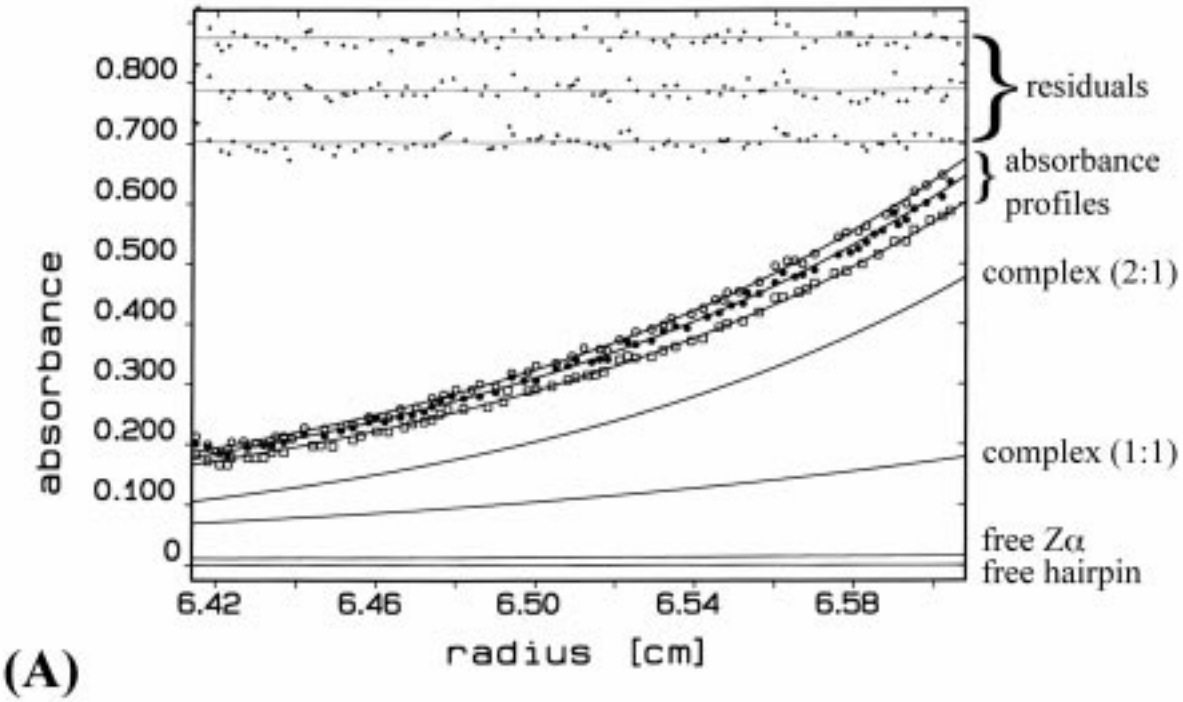
connected through a T_4 hairpin loop. Such a hairpin has been previously found to crystallize in the Z-DNA conformation [171].

Two Zα domains bind to six d(CG) basepairs

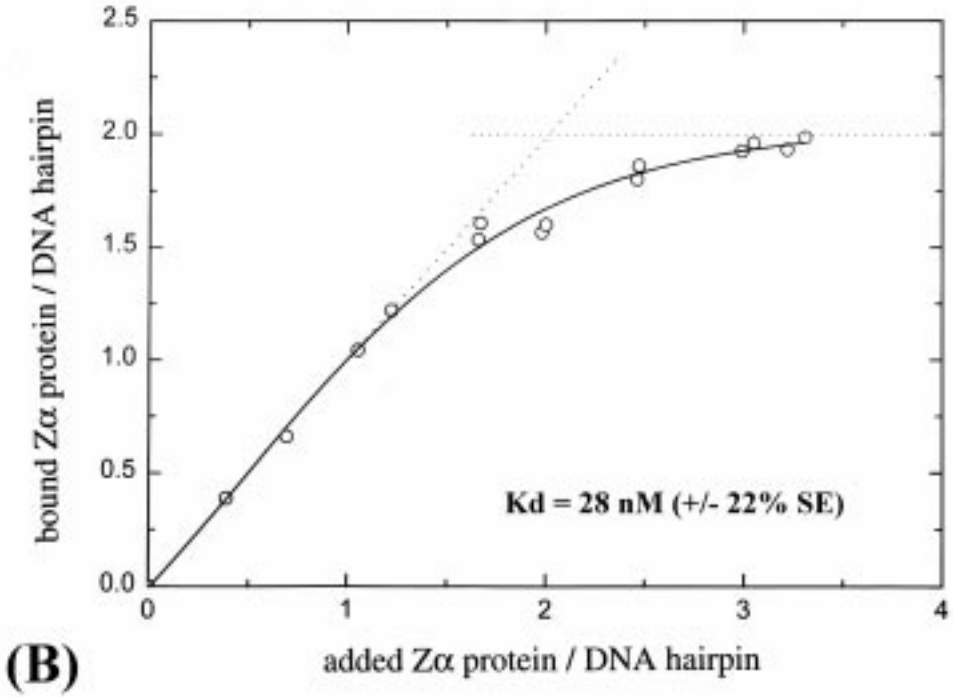
Previous gel shift experiments suggested that the Zα domain binds as a dimer to multiple binding sites in $d(CG)_{35-45}$ polymers [15]. Here the binding of Zα to the $d(CG)_3T_4(CG)_3$ hairpin was measured by analytical ultracentrifugation at various Zα/hairpin ratios. In the equilibrium ultracentrifugation experiment, bound and unbound Zα and hairpin are distributed in the gravity field of the ultracentrifuge on the basis of their different molecular weights. The radial concentration profiles of these species are determined from their UV absorbance between 255 and 285 nm. Assuming that the four species, free DNA, free Zα, Zα/hairpin and $(Z\alpha)_2$/hairpin complex, can be present in solution, least square fitting of the radial concentration profiles allows one to calculate the partial concentration profile of each species. At a molar Zα/hairpin ratio of 1.7 the mathematical model fits the experimental absorbance profiles well, as demonstrated by the statistical distribution of the residuals (fig. 19A). The calculated partial concentration profiles in the lower part of fig. 19A show that almost all Zα and hairpin molecules are in the bound state at a 1.7 Zα/hairpin ratio. The profiles of (1:1) and (1:2) complexes reflect the best fit to a four-component statistical binding model rather than the accurate ratio of these species, which could not be determined from our data. The sum of the partial concentrations of (1:1) and (1:2) complexes plotted against the molar ratio of Zα/hairpin (fig. 19B) shows a saturation plateau at a stochiometry of two Zα domains to one hairpin. In addition to this binding curve, we analyzed the change of the mean molecular weight of the species as a function of added protein. We found that the mean molecular weight reaches a maximum at a stochiometry of about 2, and began to fall off upon addition of excess protein [data not shown]. The maximum molecular weight is consistent with a ternary complex of two Zα domains bound to one hairpin.

It was also investigated whether the Zα domain by itself is purely monomeric in solution or whether it exists in a dimeric state that functions as a preequilibrium for binding to cognate DNA. By analytical ultracentrifugation of the protein alone, it was found that the molecular weight of Zα is 9400, which is in excellent agreement with the calculated molecular weight of the Zα monomer of 9409. Thus, free Zα is >95% monomeric in solution. Taken together, these data demonstrate that two Zα domains bind to six d(CG) bp.

fig. 19 **Analytical ultracentrifugation of Zα/hairpin complex (see next page). (A)** The analysis of radial absorbance profiles is shown for Zα and hairpin at a molar ratio of 1.7. The fit of the absorbance profiles at 260, 265, 270 nm (open squares, closed circles and open circles, respectively) to a 4 component exponential function is portrayed in the middle part of the figure. The top part demonstrates that the corresponding residuals (as defined in the methods) are statistically distributed. The bottom part illustrates the resulting partial radial concentration profiles for each of the 4 species, free DNA, free Zα, 1:1 and 1:2 complex. At this Zα/hairpin ratio, complex predominates. **(B)** The ratios of bound Zα/$d(CG)_3T_4(CG)_3$-hairpin, calculated from the radial concentration profiles in the ultracentrifuge cell, are plotted with open circles for each Zα/hairpin stochiometry tested. The data of two independent titration experiments are shown as pairs of open circles with similar x and y values. The initial slope and the saturation plateau of the binding curve are marked by dotted lines, crossing each other at a stochiometry of 2 Zα domains/hairpin. The K_d of 28 nM (+/- 22% SE) was determined directly from the radial concentration profiles.


absorbance
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0
6.42
6.46
6.50
6.54
6.58
radius [cm]
residuals
absorbance profiles
complex (2:1)
complex (1:1)
free Zα
free hairpin

(A)


bound Zα protein / DNA hairpin
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
added Zα protein / DNA hairpin
Kd = 28 nM (+/- 22% SE)

(B)

In a titration experiment using CD spectroscopy, we tested whether the conformational change from B- to Z-DNA upon binding of Zα is in accordance with the binding stochiometry observed by ultracentrifugation. The CD spectrum (fig. 20) shows that the hairpin adopts a conventional B-DNA conformation in the absence of Zα protein. The molar ellipticities around 253 and 296.5 nm dramatically altered upon addition of increasing amounts of Zα. This change is characteristic of the transition from B- to Z-DNA and is similar to that obtained at high ionic strengths, which are known to produce Z-DNA with alternating $d(CG)_n$ sequences [61]. The CD curves at stochiometries of 2, 3 and 4 Zα domains per hairpin superimpose, indicating that the left-handed DNA conformation remains unchanged upon overtitration. At wavelengths below 250 nm, where the CD signal of the protein is predominant, the 3 CD curves split reflecting increasing protein concentration. This data demonstrates that the binding curve reaches a plateau at a stochiometry of two Zα domains to one hairpin, and that binding entails conformational change from B- to Z-DNA. The saturation point at a ternary complex of two Zα domains and one hairpin is identical with the results from the ultracentrifugation experiments. A similar binding stochiometry was found in a previous study, using a different Zα construct,

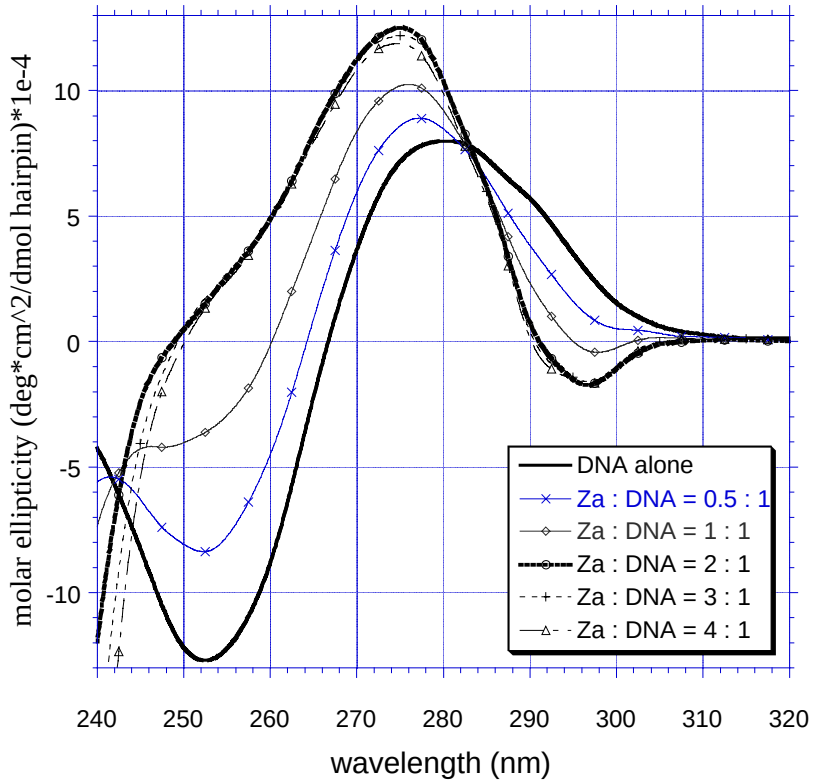


fig. 20 **CD titration of Zα/hairpin complex.** The CD spectra at stochiometries of 0.5, 1, 2, 3 and 4 Zα-domains/$d(CG)_3T_4(CG)_3$-hairpin show a large alteration of the molar ellipticities at 253 and 296.5 nm with increasing amounts of Zα, reaching saturation at a stochiometry of 2 Zα domains/hairpin. The CD spectra of hairpin DNA alone (thick black line) is characteristic of B-DNA, whereas the CD spectrum at saturation (thick dotted line) is characteristic of Z-DNA. The isodichroic point at 282.5 nm suggests a two state conformational transition between B- and Z-DNA. The protein itself has a strong negative ellipticity below 250 nm.

which did not show a well defined saturation [169]. Thus, despite the shortness of the binding site on the hairpin, two Zα domains can be accommodated.

Affinity of Zα to $d(CG)_3T_4(CG)_3$ and polyd(CG)

The affinity of Zα to the $d(CG)_3T_4(CG)_3$ hairpin was also determined by analytical ultracentrigation. A single mean K_d of 28 nM (+/- 22% SE) was calculated from the binding curve in fig. 19B using a four-component statistical binding model. The K_d's for (1:1) and (1:2) complexes are almost identical suggesting that the hairpin harbors two independent binding sites for Zα. This finding is consistent with the crystal structure of the $(Z\alpha)_2$/Z-DNA complex showing that the two Zα domains bound to a six bp d(CG) duplex do not touch each other [6].

In order to compare the double binding site on the hairpin with a contiguous string of binding sites on a $d(CG)_n$ polymer, the affinity of Zα to polyd(CG) was determined by surface plasmon resonance. The polyd(CG) ligand was previously stabilized in the Z-DNA conformation by chemical bromination [3,16]. The binding curves for protein injections of concentrations around the equilibrium dissociation constant are shown in fig. 21A. They encompass an association phase of 180 s and a dissociation phase of 180 s. After a concentration-dependent association time, all curves reached a steady-state response. The steady state response at each concentration was used to calculate the binding constant by non-linear least square fitting to a Langmuir binding curve (A + B = AB) (fig. 21B). A K_d of 29 nM (± 8% SE) was found for the Zα/polyd(CG) interaction. The comparison with the K_d of the hairpin demonstrates that Zα has, within the accuracy of our affinity measurements, the same affinity to the hairpin as to polyd(CG), indicating that six d(CG) bp are sufficient to form an intact binding site for Zα. Moreover, the affinity of Zα to the brominated polymer does not appear to be modulated by nearest neighbor effects or the effects of bromination.

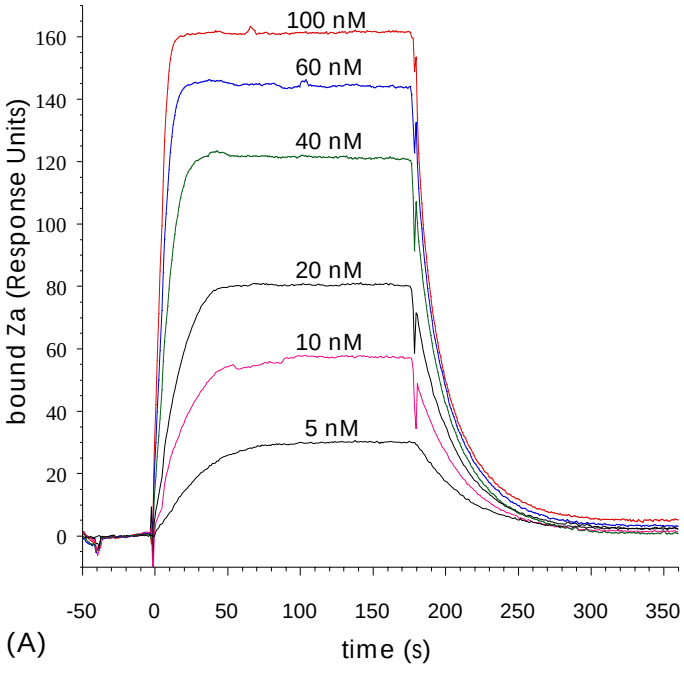


fig. 21(A) **BIAcore of Zα binding to brominated polyd(CG).** BIAcore sensograms of injections of 5, 10, 20, 40, 60 and 100 nM Zα protein start at time 0 s and reached a concentration-dependent steady-state response after a short association phase. The spikes at the beginning of the dissociation phase (at 180 s) arise from offset artifacts introduced by background subtraction from a second uncoated chip over which the protein solution was also passed. Zα dissociation is almost complete indicating a reversible reaction.

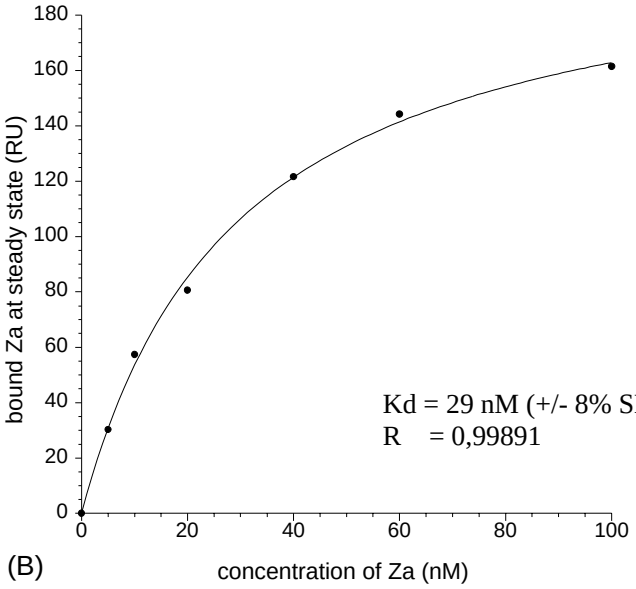


fig. 21(B) **BIAcore of Zα binding to brominated polyd(CG).** Steady state analysis by least-square fitting against a Langmuir isotherm yields a K_d of 29 nM for wild-type Zα.

Binding and dimerization of Zα-C125S mutant

In previous gel shift studies, the Zα-C125S mutant exhibited a strongly reduced affinity to Z-DNA as compared to wild-type [15]. Furthermore, the bandshift activity of wild-type Zα was diminished when the experiment was performed in the presence of 100 mM β-mercaptoethanol. These data suggested that a disulfide bond between the cysteines at position 125 of two Zα domains might stabilize the Zα/Z-DNA interaction under the conditions of the gel shift assay. In order to investigate the requirement of this potential disulfide bond for tight binding to Z-DNA, we determined the affinity constants of the Zα-C125S mutant by both analytical ultracentrifugation and surface plasmon resonance. In agreement with the results for wild-type Zα, we found by ultracentrifugation that Zα-C125S is monomeric in the absence of Z-DNA, and that two mutant domains bind to one hairpin (fig. 22A). The mean K_d was measured to be 30 nM (+/- 25% SE). The surface plasmon resonance measurements yielded a K_d of 23 nM (+/- 14% SE) (fig. 22B). The good agreement between these K_d data and those obtained for wild-type Zα shows that the affinity of Zα to Z-DNA under reducing conditions is unaffected by the C125S substitution. Moreover, the Zα domain forms a tight $(Z\alpha)_2$/hairpin complex without a disulfide bond consistent with the absence of inter-Zα contacts in the crystal structure of the $(Z\alpha)_2$/Z-DNA complex [6]. However, under less strongly reducing conditions, the formation of a disulfide bond at position 125 may tether two Zα domains. This would stabilize the Zα/Z-DNA complex and slow its breakdown in bandshift experiments thereby accounting for the differential binding affinities between Zα-C125S and wild-type, as seen in such studies.

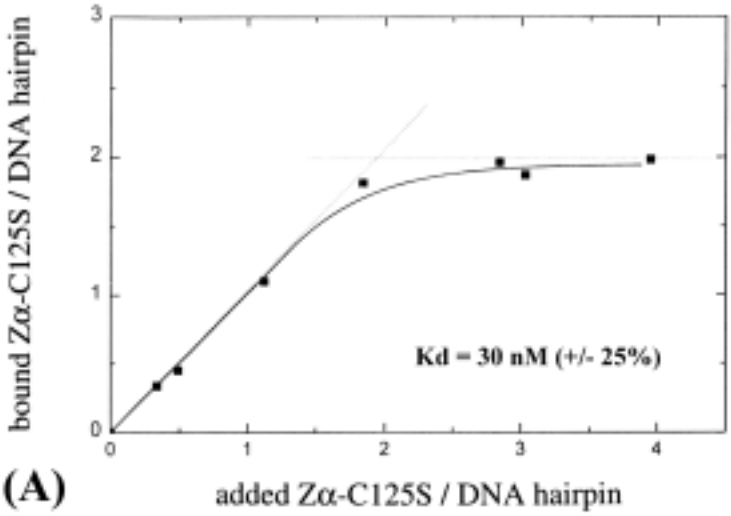
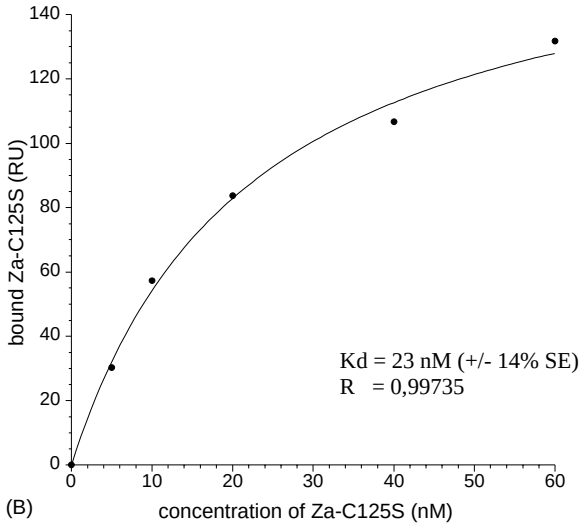


fig. 22 **Analytical ultracentrifugation and BIAcore of Zα-C125S/hairpin complex** **(A)** The binding curve of the mutant Zα-C125S with the $d(CG)_3T_4(CG)_3$ hairpin was determined by analytical ultracentrifugation (display analogous to fig. 19B). The K_d of 30 nM (+/- 25% SE) and the stochiometry of the mutant is identical to wild-type.



(B) The BIAcore steady-state binding of Zα-C125S to brominated polyd(CG) yielded a K_d of 23 nM (+/- 14% SE), which is about the same as wild-type (display analogous to fig. 21B).

Summary

The Zα domain of the human RNA editing enzyme, double-stranded RNA deaminase I (ADAR1), binds to left-handed Z-DNA with high affinity. By analytical ultracentrifugation and CD spectroscopy, it was found that two Zα domains bind to one $d(CG)_3T_4(CG)_3$ hairpin which contains a stem of six basepairs in the Z-DNA conformation. These results in solution are consistent with the crystal structure of two Zα domains complexed with a duplex of six d(CG) bp which shows that the two Zα domains bind on opposite sides without contacting each other. Both wild-type Zα and a C125S mutant show a mean dissociation constant of 30 nM as measured by surface plasmon resonance and analytical ultracentrifugation. Our data suggest that short (≥ 6 basepair) segments of Z-DNA within a gene are able to recruit two ADAR1 enzymes to that particular site. This may ensure rapid editing of multiple nascent pre-mRNA substrates prior to the removal of introns by splicing if many transcription complexes work simultaneously on that gene.