A 6 bp Z-DNA hairpin binds two Zα domains from the human RNA editing enzyme ADAR1

Markus Schade[a,b], Joachim Behlke[c], Ky Lowenhaupt[a], Alan Herbert[a], Alexander Rich[a,*], Hartmut Oschkinat[b,1]

[a]*Department of Biology, Massachusetts Institute of Technology (MIT), 77 Mass. Av., Cambridge, MA 02139, USA*
[b]*Forschungsinstitut für molekulare Pharmakologie, Alfred-Kowalke-Str. 4, 10315 Berlin, Germany*
[c]*Max Delbrück Center for Molecular Medicine, 13122 Berlin, Germany*

Abstract The Zα domain of the human RNA editing enzyme double-stranded RNA deaminase I (ADAR1) binds to left-handed Z-DNA with high affinity. We found by analytical ultracentrifugation and CD spectroscopy that two Zα domains bind to one $d(CG)_3T_4(CG)_3$ hairpin which contains a stem of six base pairs in the Z-DNA conformation. 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 bp) segments of Z-DNA within a gene are able to recruit two ADAR1 enzymes to that particular site.
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Key words: RNA editing; ADAR1; Zα; Z-DNA; Helix-turn-helix; Ultracentrifugation

1. Introduction

RNA editing in mammals alters codons in mRNA through site-specific adenosine-to-inosine (A→I) deamination [1]. A→I editing of the subunit B of neural AMPA-sensitive glutamate receptors (GluR-B) substitutes a glutamine for an arginine in the ion channel of the receptor thereby reducing its calcium permeability [2]. Knock-out mice deficient in RNA editing of GluR-B had epileptic seizures and died 3 weeks after birth, suggesting that editing of GluR-B is essential for normal brain function [3]. A→I editing of serotonin 2C receptors alters three adjacent amino acids in the second intracellular loop, resulting in a 10–15-fold decreased G protein coupling efficacy [4].

Double-stranded RNA deaminases type I and II (ADAR1/2) catalyze A→I editing. ADAR1, but not ADAR2, contains the N-terminal domain Zα, which binds specifically to left-handed Z-DNA [5]. Z-DNA can form readily from B-DNA under conditions of negative supercoiling [6]. In vivo, negative supercoiling can be transiently generated 5′ to a moving RNA polymerase [7]. Transcription-dependent Z-DNA formation in vivo has been demonstrated in both prokaryotes and euka-

ryotes and in metabolically active, agarose-embedded nuclei from a primary liver cell line [6]. Z-DNA forms preferentially in sequences of alternating pyrimidine/purine with $d(CG)_n$ performing best. Consequently, the transient, sequence- and supercoiling-dependent Z-DNA formation adjacent to a moving RNA polymerase may generate a binding site for Zα, thereby targeting the catalytic activity of ADAR1 into the vicinity of certain nascent pre-mRNAs [6]. Thus, Zα may contribute to the selection of editing sites when sequences within a gene form Z-DNA.

Zα is a 63 residue domain with an (α+β) helix-turn-helix (α+βHTH) fold, showing structural similarity to other members of this widespread family of B-DNA binding protein domains [8]. It has been shown by several biochemical and spectroscopic studies in vitro [9–11], and by the crystal structure of Zα bound to substrate DNA [12] that the DNA complexed with Zα adopts the left-handed Z-conformation. Here we have used a defined hairpin substrate, $d(CG)_3T_4(CG)_3$, to determine the binding constant and stoichiometry of Zα bound to Z-DNA in solution. By analytical ultracentrifugation and circular dichroism (CD) spectroscopy, we 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. Furthermore, we found a virtually identical K_d and stoichiometry for the C125S mutant of Zα, which was investigated by NMR spectroscopy and which showed a distinct binding behavior in previous bandshift experiments [10]. Taken together, this work demonstrates that the formation of a compact ternary $(Zα)_2$/Z-DNA complex requires a binding site of no more than six base pairs. In contrast, B-DNA binding α+βHTH proteins usually require a recognition site of eight or more base pairs.

2. Materials and methods

2.1. Protein preparation and site-directed mutagenesis
The Zα domain (residues 119–200) of human ADAR1 (GenBank accession number U10439) was expressed as a fusion protein with a N-terminal $(His)_6$ tag from a pET-21a vector (Novagen) in *Escherichia coli* strain HM174(DE3) (Novagen), utilizing the T7 expression system. Harvested bacteria were resuspended in binding buffer (20 mM Tris-HCl pH = 8, 150 mM NaCl, 0.125 mM PMSF, 10 mM β-mercaptoethanol) and lysed using a French press. The *E. coli* lysate was passed over a $(His)_6$ tag affinity column (TALON Metal Affinity Resin, Clontech, Palo Alto, CA, USA), and the Zα fusion protein eluted using a 0–300 mM imidazole gradient. After dialysis against binding buffer, the $(His)_6$ tag was removed by thrombin digestion at room temperature overnight. For the second purification step, the Zα protein was dialyzed into 50 mM HEPES pH = 7.4, 50 mM NaCl,

1 mM EDTA, 1 mM DTT, 0.125 mM PMSF and loaded on a cation exchange chromatography column (Mono S 5/5, Pharmacia, Uppsala, Sweden). The protein was eluted with a 50–1000 mM NaCl gradient, yielding homogeneous protein of wild-type molecular weight, as indicated by SDS-gel analysis and MALDI-TOF mass spectroscopy.

The Zα-C125S mutant was constructed by PCR-based site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) in the pET-21a vector harboring the Zα insert. The mutant plasmid was grown in the cloning strain *E. coli* DH-5α. The mutant protein was expressed and purified as described above.

2.2. BIAcore analysis

Binding kinetics were measured by surface plasmon resonance on a BIAcore 2000 instrument as described [9]. Briefly, 270 response units (RU) of polyd(CG) stabilized in the Z-DNA conformation by chemical bromination was immobilized on the chip surface through a biotin linkage. Protein solutions at different concentrations were passed over the chip surface with a flow rate of 20 µl/min in phosphate buffer (pH = 7.4), 137 mM NaCl, 1 mM EDTA and 1 mM DTT at 25°C. The baseline obtained by traversing the protein solution in line over a second chip surface lacking immobilized DNA was subtracted from each experimental curve. The Zα protein did not bind to a random sequence B-DNA polymer of the same size and immobilized at the same density as the Z-DNA probe [9]. Binding curves were analyzed both by least square fitting to Langmuir isotherms and by time-resolved global analysis (BIAevaluation software 3.0 with mass transport correction, BIAcore Inc.) to cross-validate the K_d results. The apparent on-rates for Zα and Zα-C125S were faster than 10^6 M^{-1} s^{-1} and are affected by mass-transport artifacts [13]. Therefore, for calculation of the K_d, steady-state fitting to Langmuir isotherms was considered to be more robust.

2.3. Ultracentrifugation

Analytical ultracentrifugation experiments were performed on a Beckman XL-A ultracentrifuge in 10 mM Na-phosphate buffer (pH = 5.5) and 137 mM NaCl. The buffer for the wild-type sample was supplemented with 100 mM β-mercaptoethanol. Sedimentation velocity experiments were performed in standard double sector cells at 20°C. Equilibrium experiments were performed at 10°C in six channel cells making use of the overspeed technique. Here, the samples were centrifuged for 2 h at 24 000 rpm (overspeed step), and were subsequently spun at an equilibrium speed of 20 000 rpm for 30–32 h (equilibrium step). The radial concentration profile of the d(CG)₃T₄(CG)₃ hairpin and of the Zα protein was measured by UV absorption at six equidistant wavelengths ranging from 255 to 285 nm. The approximate extinction coefficients were calculated from the sequences as described for DNA [14] and for protein [15].

Data collection and analysis of the four component system, free hairpin, free Zα, complex Zα/hairpin and (Zα)₂/hairpin, required a two-step approach. First, the molecular weights of both the hairpin and the Zα protein alone were determined by fitting the radial concentration profiles in sedimentation velocity and equilibrium experiments as described [16,17]. These data were used to reduce the number of variables in the equation describing the four component system. Second, the concentration profiles of the complex were measured in a titration series with increasing protein concentrations. Protein concentrations from 0.7 to 6.0 µM were tested in the presence of 1.8 µM hairpin. The experimental concentration profiles were fitted to the sum of four exponential functions (one for each of the four species) as described [16,18]. This global fitting was performed for the various Zα/hairpin ratios of the titration yielding the K_d. The resulting ratios of bound Zα/hairpin yielded a binding curve (Fig. 1B), from which the binding stoichiometry was obtained. Since the partial concentrations for the Zα/hairpin and (Zα)₂/hairpin complex in the titration are well described by a statistical binding model assuming identical binding sites, a single mean K_d for both binding steps was determined. The robustness and accuracy of this fitting procedure has been shown for other binding equilibria [16,18].

2.4. CD spectroscopy

The CD titration of 10 µM d(CG)₃T₄(CG)₃ hairpin with increasing amounts of Zα protein was recorded in 10 mM Na-phosphate buffer (pH = 5), 75 mM NaF, 0.1 mM NaN₃ at 21°C on a Jasco J-720 CD spectrometer using a cuvette with a 10 mm pathlength. After 15 min of thermal equilibration, data were acquired at 0.1 nm resolution with

a scan speed of 50 nm/min. The mean of six data accumulations was smoothed with a third order polynomial function. A positive control of d(CG) oligomers in the Z conformation at high ionic strength (4 M NaCl) is shown elsewhere [10].

3. Results and discussion

In this work we determined the stoichiometry and affinity of the Zα domain to a d(CG)₃T₄(CG)₃ hairpin, which contains six base pairs of alternating d(CG) substrate DNA. In contrast, previous studies [9] utilized polyd(CG) to determine the affinity of Zα to Z-DNA. The two d(CG)₃ strands of this hairpin were tethered through a T₄ loop, which increases the melting temperature by 40°C ensuring that a stable duplex
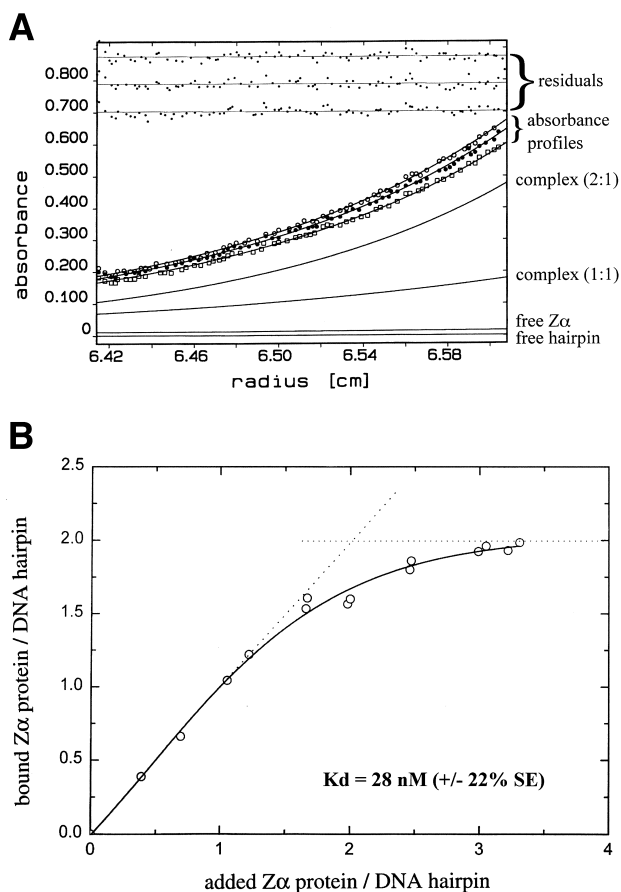

Fig. 1. Analytical ultracentrifugation of Zα/hairpin complex. A: The analysis of radial absorbance profiles is shown for Zα and hairpin at a molar ratio of 1.7. The fit of the absorbance profiles at 260, 265, 270 nm (open squares, closed circles and open circles, respectively) to a four component exponential function is portrayed in the middle part of the figure. The top part demonstrates that the corresponding residuals (as defined in Section 2) are statistically distributed. The bottom part illustrates the resulting partial radial concentration profiles for each of the four 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)₃T₄(CG)₃ hairpin, calculated from the radial concentration profiles in the ultracentrifuge cell, are plotted with open circles for each Zα/hairpin stoichiometry 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 stoichiometry of two Zα domains/hairpin. The K_d of 28 nM (± 22% S.E.M.) was determined directly from the radial concentration profiles.

stem is present at room temperature [19]. Analytical ultracentrifugation verified that d(CG)$_3$T$_4$(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)$_3$T$_4$(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$_2$O 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)$_3$T$_4$(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 [20].

3.1. Two Zα domains bind to six d(CG) base pairs

Previous gel shift experiments suggested that the Zα domain binds as a dimer to multiple binding sites in d(CG)$_{35-45}$ polymers [10]. Here the binding of Zα to the d(CG)$_3$T$_4$(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 (Zα)$_2$/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. Fig. 1A exemplifies at a molar Zα/hairpin ratio of 1.7 that the mathematical model fits the experimental absorbance profiles well, as demonstrated by the statistical distribution of the residuals. The calculated partial concentration profiles in the lower part of Fig. 1A 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. 1B) shows a saturation plateau at a stoichiometry 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 stoichiometry 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.

We 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) base pairs.

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 stoichiometry observed by ultracentrifugation. The CD spectrum (Fig. 2) shows that the hairpin adopts a conventional B-DNA confor-
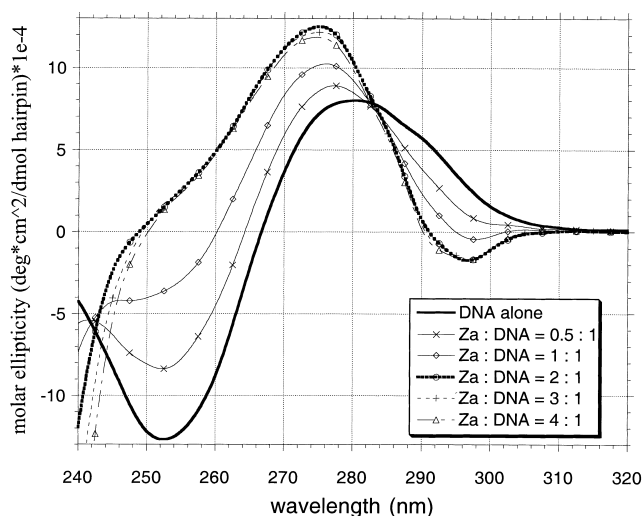

Fig. 2. CD titration of Zα/hairpin complex. The CD spectra at stoichiometries of 0.5, 1, 2, 3 and 4 Zα domains/d(CG)$_3$T$_4$(CG)$_3$ hairpin show a large alteration of the molar ellipticities at 253 and 296.5 nm with increasing amounts of Zα, reaching saturation at a stoichiometry of two Zα domains/hairpin. The CD spectrum 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.

mation 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 [6]. The CD curves at stoichiometries of two, three and four 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 three CD curves split reflecting increasing protein concentration. These data demonstrate that the binding curve reaches a plateau at a stoichiometry 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 stoichiometry was found in a previous study, using a different Zα construct, which did not show a well defined saturation [11]. Thus, despite the shortness of the binding site on the hairpin, two Zα domains can be accommodated.

3.2. Affinity of Zα to d(CG)$_3$T$_4$(CG)$_3$ and polyd(CG)

The affinity of Zα to the d(CG)$_3$T$_4$(CG)$_3$ hairpin was also determined by analytical ultracentrifugation. A single mean K_d of 28 nM (±22% S.E.M.) was calculated from the binding curve in Fig. 1B using a four component statistical binding model. The K_d values 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 (Zα)$_2$/Z-DNA complex showing that the two Zα domains bound to a six bp d(CG) duplex do not touch each other [12].

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 [9,21]. The binding curves for protein injections of concentrations around the equilibrium dissociation constant are shown in Fig. 3A. 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 ($A+B=AB$). A K_d of 29 nM ($\pm 8\%$ S.E.M.) 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) base pairs are sufficient to form an intact binding site for Zα. Moreover, the affinity
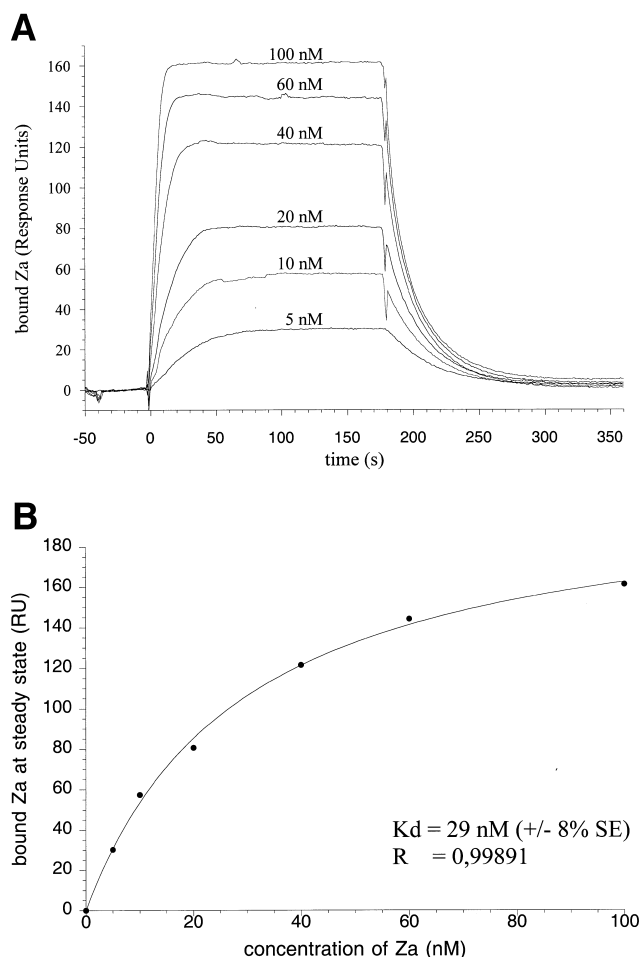

A

B

Fig. 3. BIAcore of Zα binding to brominated polyd(CG). A: BIAcore sensograms of injections of 5, 10, 20, 40, 60 and 100 nM Zα protein start at time 0 s and reach 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. B: Steady-state analysis by least square fitting against a Langmuir isotherm yields a K_d of 29 nM for wild-type Zα.
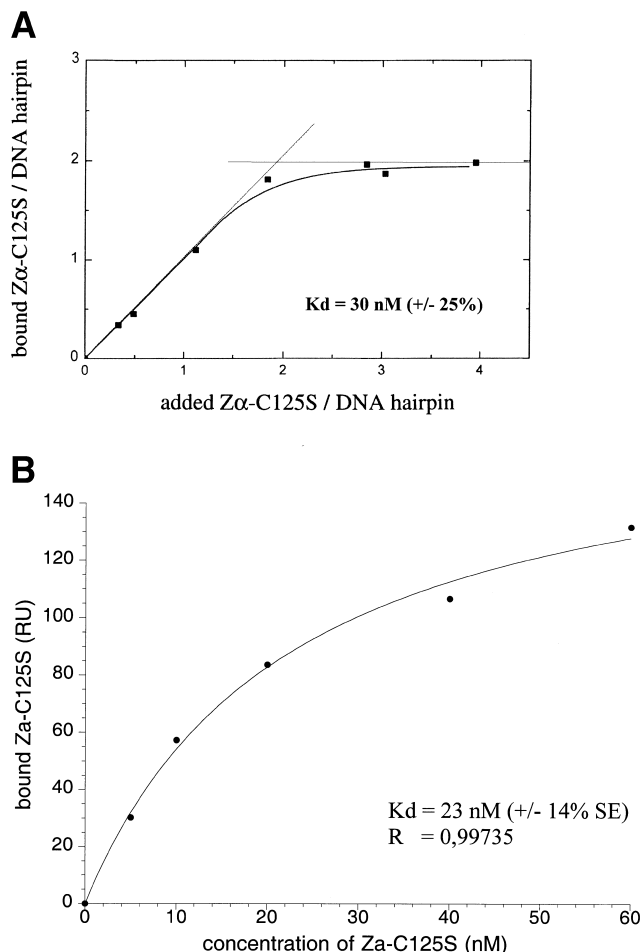

A

B

Fig. 4. 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. 1B). The K_d of 30 nM ($\pm 25\%$ S.E.M.) and the stoichiometry 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 ($\pm 14\%$ S.E.M.), which is about the same as wild-type (display analogous to Fig. 2B).

of Zα to the brominated polymer does not appear to be modulated by nearest neighbor effects or the effects of bromination.

3.3. Binding and dimerization of Z_α-C125S mutant

In previous gel shift studies, the Zα-C125S mutant exhibited a strongly reduced affinity to Z-DNA as compared to wild-type [10]. 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. 4A). The

mean K_d was measured to be 30 nM ($\pm 25\%$ S.E.M.). The surface plasmon resonance measurements yielded a K_d of 23 nM ($\pm 14\%$ S.E.M.) (Fig. 4B). 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 (Zα)$_2$/hairpin complex without a disulfide bond consistent with the absence of inter-Zα contacts in the crystal structure of the (Zα)$_2$/Z-DNA complex [12]. 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.

3.4. Conclusion

We demonstrated by analytical ultracentrifugation that free Zα is monomeric in solution and that two Zα domains bind to two independent binding sites on one Z-DNA hairpin containing a 6 bp d(CG) stem. Our results in solution are consistent with the crystal structure of two Zα domains complexed with a duplex of six d(CG) base pairs which shows that the two Zα domains bind on opposite sides without contacting each other. Thus, it is possible that two or more ADAR1 enzymes are targeted to a short (≥ 6 bp) Z-DNA forming sequence in an actively transcribed gene. 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.

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