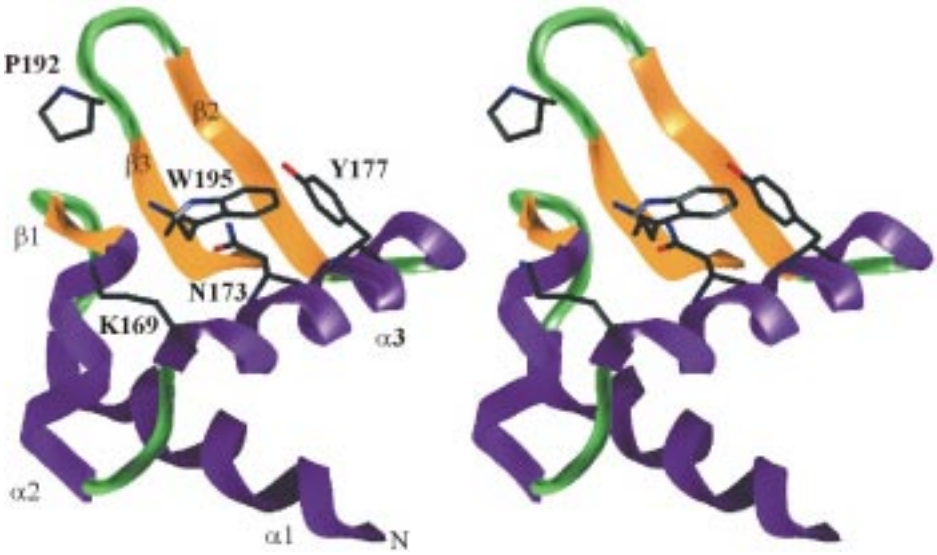
Structure and Function of the Z-DNA binding protein domain Zα



The solution structure of the Zα domain highlighting the most important Z-DNA contacting residues, as identified by scanning mutagenesis. The five alanine mutants, K169, N173, Y177, P192 and W195, showed the most dramatic reduction in affinity (ΔΔG) to Z-DNA in quantitative binding assays using surface plasmon resonance (BIAcore) spectroscopy.

Aim of this work

Adenosine to inosine RNA editing in mammals changes the genetic information on the pre-mRNA level to produce proteins with modified function. To date, this has been observed for various subunits of AMPA- and kainate-sensitive glutamate receptors [1] and for serotonin-2C receptors [2] in the central nervous system. A candidate enzyme for catalyzing these RNA editing events, double-stranded RNA deaminase I (ADAR1), contains the N-terminal domain Zα, which specifically binds to left-handed Z-DNA with high affinity in vitro, but whose function *in vivo* is unknown [3]. Zα is the first naturally occurring Z-DNA binding protein domain raising the question of how a protein can specifically recognize the left-handed conformation of DNA.

This Ph.D. thesis is aimed at exploring the functional and structural aspects of the interaction between Zα and Z-DNA. After the description of the experimental methods used for this work in chapter 1, the reader is introduced to protein/B-DNA interactions, to the biology of Z-DNA and to RNA editing in chapter 2. Chapter 3 centers on the stochiometry and the binding constant of the Zα/Z-DNA interaction which was investigated by analytical ultracentrifugation, surface plasmon resonance and CD spectroscopy using a defined hairpin substrate, $d(CG)_3T_4(CG)_3$. 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 [4]. The equilibrium dissociation constant (K_d) is 30 nM for one monomer, as measured by both analytical ultracentrifugation and surface plasmon resonance. Thus, the formation of a compact ternary $(Z\alpha)_2$/Z-DNA complex requires a binding site of no more than six basepairs.

In chapter 4, alanine scanning mutagenesis on Zα is used to identify residues that are pivotal for binding to Z-DNA. It was found that such residues cluster on one side of helix α3, making it likely that this area mediates Z-DNA contacts [5]. In addition, residues in α1, α2 and α3 were identified that probably form a hydrophobic core essential for protein stability. These data indicate that Zα shows structural similarity to (α+β)-helix-turn-helix DNA binding proteins, such as HNF-3γ and histone H5, in which α3 is used as a recognition helix. The mutational analysis is of particular importance because it paves the way for further functional studies, such as exploring the role of Zα *in vivo* by using loss-of-function mutants.

Chapter 5 describes the determination of the solution structure of free Zα whose interaction surface with the 6-basepair $d(CG)_3T_4(CG)_3$ hairpin is mapped by 2D ^{15}N-HSQC NMR spectroscopy in chapter 6. These data allow a structural analysis of functional effects observed in the aforementioned mutational investigation. It was found that the interaction surface mapped in solution agrees well with the crystal structure of Zα complexed with Z-DNA [6]. The structure of Zα free in solution undergoes only minor conformational changes upon binding Z-DNA. Not only is the overall structure the same, but unexpectedly, most Z-DNA contacting residues are prepositioned in free Zα to fit Z-DNA [7].

The Zα domain shows close structural similarity to (α+β) helix-turn-helix DNA binding proteins which exclusively bind to right-handed B-DNA [5]. In chapter 7, this study examines why Zα preferentially binds Z-DNA rather than B-DNA despite its high structural homology to (α+β) helix-turn-helix B-DNA binding proteins. The comparison of Zα with the crystal structures of four homologous protein/B-DNA complexes suggests that binding of Zα to B-DNA is disfavored by steric hindrance.

ACKNOWLEDGMENTS

I wish to thank Prof. Alexander Rich and Prof. Günter Maaß for their supervision and kind support. I am particularly grateful to Dr. Alan Herbert for teaching me fundamental techniques of molecular biology with unparalleled enthusiasm, commitment and patience. He guided and supported this project from the very beginnings all the way through. I also want to thank Dr. Ky Lowenhaupt for critical discussion, helpful advice and thorough reviewing of several manuscripts. Furthermore, I wish to thank Mrs. Carolyn Beckman-Stitson for a warm welcome in the laboratory of Prof. Rich and advice in innumerous administrative respects. I express special thanks to Dr. Christopher Turner for his exceptional patience, humor and friendship while teaching me the principles and practice of NMR spectroscopy.

I am especially grateful to Prof. Hartmut Oschkinat for scientific and financial support and for giving me the opportunity to complete my Ph.D. thesis in his laboratory. Further thanks go to Dr. Peter Schmieder for practical and scientific assistance in many aspects of modern protein NMR spectroscopy. I am also grateful to Dr. Ronald Kühne for instant help with restrained molecular dynamics calculations and preparation of glossy figures. Furthermore, I wish to thank my other colleagues in the Oschkinat group for multifaceted assistance and helpful suggestions.

Most of all, I am indebted to my parents and my entire family who comforted me and provided moral support all along.

This Ph.D. thesis was supported by a fellowship from the Boehringer Ingelheim Fonds for biomedical research (Stuttgart, Germany).

List of Publications containing parts of this Ph.D. thesis

1) Schade, M.; Turner, C.J.; Kühne, R.; Schmieder, P.; Lowenhaupt, K.; Herbert, A.; Rich, A. and Oschkinat, H. „The solution structure of the Zα domain of the human RNA editing enzyme ADAR1 reveals a prepositioned binding surfce for Z-DNA“ (1999) *Proc. Natl. Acad. Sci. USA,* **96**, 12465-12470.

2) Schade, M.; Behlke, J.; Lowenhaupt, K.; Herbert, A.; Rich, A. and Oschkinat, H. „A 6 bp Z-DNA hairpin binds two Zα domains from the human RNA editing enzyme ADAR1„ (1999) *FEBS Letters*, **458**, 27-31.

3) Schade, M.; Turner, C.J.; Lowenhaupt, K.; Rich, A. and Herbert, A. „Structure/function analysis of the Z-DNA binding domain Zα of dsRNA adenosine deaminase type I reveals similarity to the (α+β) family of helix-turn-helix proteins„ (1999) *EMBO J.*, **18**, 470-479.

4) Herbert, A.; Schade, M.; Lowenhaupt, K.; Alfken, J.; Schwartz, T.; Shlyakhtenko, L.S.; Lyubchenko, Y.L. and Rich, A. „The Zα domain from human ADAR1 binds to the Z-DNA conformer of many different sequences„ (1998) *Nucleic Acid Res.*, **26**, 3486-3493.

Contributions to Scientific Conferences

1) Markus Schade, „Structure/function analysis of the Z-DNA binding domain Zα of dsRNA adenosine deaminase type I reveals similarity to the (α+β) family of helix-turn-helix proteins“, 2nd Franco-German NMR meeting of the GDCh NMR Fachgruppe in Obernai, France, from 27.-30. September 1998 (Oral presentation)

2) Markus Schade, „Solution structure of the first Z-DNA binding protein domain Zα of the human RNA editing enzyme ADAR1”, 37th IUPAC Congress and 27th GDCh General Meeting“ in Berlin, Germany, from 14.-19. August, 1999 (Oral presentation)

3) Markus Schade, et al. „The solution structure of the Zα domain of the human RNA editing enzyme ADAR1 reveals a prepositioned binding surfce for Z-DNA“, Meeting of the GDCh NMR-Fachgruppe in Würzburg, Germany from 29.9.-2.10.1999 (Poster presentation and Reception of a „R.R. Ernst Stipendium“)

TABLE OF CONTENTS

ABBREVIATIONS

Material and Methods:

BSA	Bovine serum albumin
CD	Circular Dichroism
DSS	2,2-Dimethyl-2-silapentane-5-sulfonate, sodium salt
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphic acid])
Igepal	Non-ionic detergent
IPTG	Isopropyl-β-D-thio-galactopyranoside
LB	Luria Broth
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight (mass spectroscopy)
OD	Optical density
PAGE	Polyacrylamide electrophoresis
PMSF	Phenyl-methanesulfon-fluoride
PBS	Phosphate buffered saline
rpm	Revolutions per minute
RU	Response Units (for BIAcore machine)
SDS	Sodium dodecyl sulfat
SE	Standard error
SPR	Surface plasmon resonance
Tris	Tris(-hydroxymethyl)-aminomethane
UV	Ultra violet

Biochemical terminology:

ADAR	Adenosine deaminase dsRNA-specific (formerly: dsRNA deaminase)
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxaleproprionate
APOBEC1	*apoB* mRNA editing cytidine deaminase 1
bp	basepair
cAMP	cyclo-Adenosine-mono-phosphate
CAP	Catabolite activator protein
DNA	Deoxyribonucleic acid
DtxR	Diphtheria toxin repressor
ds	double-stranded
GST	Gluthathione-S-transferase
HNF-3γ	Hepatocyte nuclear factor 3γ
HMG	High mobility group
HTH	Helix-turn-helix
K_d	Equilibrium dissociation constant
LEF-1	Lymphoid enhancer-binding factor 1
NFAT	Nuclear factor of activated T-cells

RED2	dsRNA editase 2
rmsd	root mean standard deviation
RNA	Ribonucleic acid
SRY	sex-determining region Y
TBP	TATA box binding protein

NMR terminology:

COSY	Correlation spectroscopy
FID	Free induction decay
FT	Fourier transformation
HSQC	Heteronuclear single quantum correlation
INEPT	Insensitive nucleus enhancement by polarization transfer
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser enhancement (effect) spectroscopy
ppm	Parts per million
rf	Radio frequency (pulse)
T_1	Longitudinal (= spin-lattice) relaxation time
T_2	Transverse (= spin-spin) relaxation time
TOCSY	Total correlation spectroscopy

C h a p t e r 1

MATERIALS AND METHODS

Materials

If not otherwise specified all organic and inorganic chemicals were purchased in the highest available quality from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). For enzymes and related biochemicals, the common vendor was New England Biolabs (Beverly, MA, USA).

Buffers and Solutions

Phosphate buffered saline (PBS buffer, pH 7.3):

140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4

M9 Minimal Medium (1x):

$Na_2HPO_4*7H_2O$	48 mM	12.8 g	per liter H_2O
KH_2PO_4	22 mM	3.0 g	per liter H_2O
NaCl	9 mM	0.5 g	per liter H_2O
NH_4Cl	19 mM	1.0 g	per liter H_2O
$MgSO_4$	2 mM	add as sterile solution separately	
$CaCl_2$	0.1 mM	add as sterile solution separately	
Glucose	0.4 %	add as sterile 40 % solution separately	

Suppliers

BioRad, BioRad, Melville, NY
Bruker GmbH, Rheinstetten, Germany
CalBiochem, La Jolla, CA, USA
Clontech, Palo Alto, CA, USA
Cromassie Brilliant Blue dye (Instaview universal, BDH laboratory, Poole, GB)
Dupont, Wilmington, DE, USA
GibcoBRL, Gaithersburg, MD, USA
Isotec Inc., Miamisburg, OH, USA
Kirkegaard & Perry labs, Gaithersburg, USA
Martek Biosciences Corp., Columbia, MD, USA
Millipore Corp., Bedford, MA, USA

MSI, Molecular Simulations Inc., San Diego, CA, USA
New England Biolabs, Beverly, MA, USA
Novagen, Madison, WI, USA
Pharmacia, Piscataway, NJ, USA
Pierce, Rockford, IL, USA
Sigma, St.Louis, MO, USA
SLM Instruments Inc., Urbana, IL, USA
Sorvall Instruments, Wilmington, DE, USA
Stratagene, La Jolla, CA, USA
Tripos Inc., St.Louis, MO, USA

Methods

Protein preparation

Site-specific Zα mutants were constructed by PCR-based site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) and checked by DNA sequencing. Mutant and wild-type Zα proteins containing residues 120-197 of human ADAR1 (GenBank accession no. U10439), were expressed as Glutathione-S-Transferase(GST)-fusion proteins (GST is N-terminal) in pGEX-5x1 vectors (Pharmacia) in *E.coli* strain DH-5α (GibcoBRL). The cells were grown at 37° C in 450 mL cultures of LB-medium containing 2 % glucose and 50 µg/mL ampecillin. At an optical density (OD) of 1.0 at 600 nm, the cells were induced with 1 mL 100 mM IPTG and grown for another hour. The cells were harvested by centrifugation at 6000 rpm for 30 min at 4° C (rotor SS34, Sorvall Instruments), resuspended in 10 mL phosphate buffered saline (PBS) containing 0.1 mM protease inhibitor PMSF, and lysed by French pressing (SLM Instruments) at a pressure of 950 psi. The GST-fusion protein was purified by affinity chromatography using glutathione-agarose (Sigma). The soluble fraction of the lysed cells was loaded on a 300 mg glutathione-agarose column (10 mL disposable column, Pierce) and eluted with 10 mL PBS containing 10 mM glutathione. Proteins were checked by SDS-PAGE on 15 % gels and probed for binding to Z-DNA by bandshift and Southwestern assays.

For the preparation of Zα peptides, GST-fusion proteins were digested on-column with factor Xa protease (New England Biolabs) in 20 mM HEPES (pH 8), 100 mM NaCl, 2 mM $CaCl_2$, 0.05% n-Octyl-glucopyranoside at 22° C over night. The Zα peptides were washed off the column with the same buffer at pH 7.4 containing 1 mM DTT. These Zα peptides were checked by SDS-PAGE on 18 % gels and tested for binding to Z-DNA by bandshift assays.

Key Zα mutants were further investigated by surface plasmon resonance and CD spectroscopy. These mutants and a wild-type control comprising residues 122-197 of ADAR1, were expressed in pET-28a vectors in *E.coli* strain Novablue (Novagene). The pET-28a encoded construct differs from the pGEX-5x1-derived peptides in having a stop codon after I197, deleting 12 residues encoded by the pGEX-5x1 polylinker. In addition, the pET-28a construct, which is called the short Zα construct, contains five vector-encoded residues (GSHMG) in the N-terminus compared with three residue (GIP) in the pGEX-5x1 vector construct. Proteins were purified in two steps. First, the *E.coli* lysate was passed over a $(His)_6$-tag affinity column (TALON Metal Affinity Resin, Clontech) in 20 mM Tris/HCl (pH 8), 150 mM NaCl, using a 0 - 300 mM imidazole gradient for elution. The $(His)_6$-tag was removed by thrombin digestion (CalBiochem) in the same buffer. For the second purification step, protein was loaded on a cation exchange chromatography column (Mono S 5/S, Pharmacia) in 50 mM HEPES (pH 7.4), 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.125 mM PMSF and eluted with a 25 - 1000 mM NaCl gradient, yielding homogeneous protein of wild-type molecular weight, as indicated by SDS-PAGE analysis and by MALDI-TOF mass spectroscopy.

For NMR, analytical ultracentrifugation and surface plasmon resonance measurements on wild-type and C125S mutant Zα, a different pET-28a construct was used which is referred

to as the long Zα contruct in the following text. It comprises residues 119-200 of human ADAR1 and three additional vector-encoded residues: a glycine and a serine residue at the N-terminus and an aspartic acid at the C-terminus. The protein preparation was identical to that used for the short pET-28a construct.

For isotope labeling, the pET-28a vector (residues 119-200) was transformed into *E.coli* strain HM174(DE3) (Novagen), and the bacteria were grown in M9 minimal medium containing 1 g/L $^{15}NH_4Cl$ (Isotec Inc.) and 1.5 g/L ^{13}C-glucose (Martek Biosciences). The protein purification was unchanged.

Analytical ultracentrifugation

Analytical ultracentrifugation is a widely applied technique to study the molecular weight and hydrodynamic properties of biological macromolecules in solution. In sedimentation velocity experiments, the centrifugal force moves solute macromolecules at a certain rate to the bottom of the centrifuge cell. The rate per unit centrifugal field, s, is proportional to the molecular weight and inversely proportional to the frictional coefficient, which contains information about the shape of the macromolecule [8]. In sedimentation equilibrium experiments, the centrifugal force on a macro-molecule is balanced by diffusion leading to an equilibrium distribution of the macromolecule, from which its molecular weight can be determined. If two different macromolecules that bind to each other can be discriminated in the ultracentrifuge, for instance by their UV absorbance (fig. 1), the binding constant can be determined from the analysis of their equilibrium distribution. Thus, analytical ultracentrifugation is a powerful technique to investigate binding equilibria of unknown

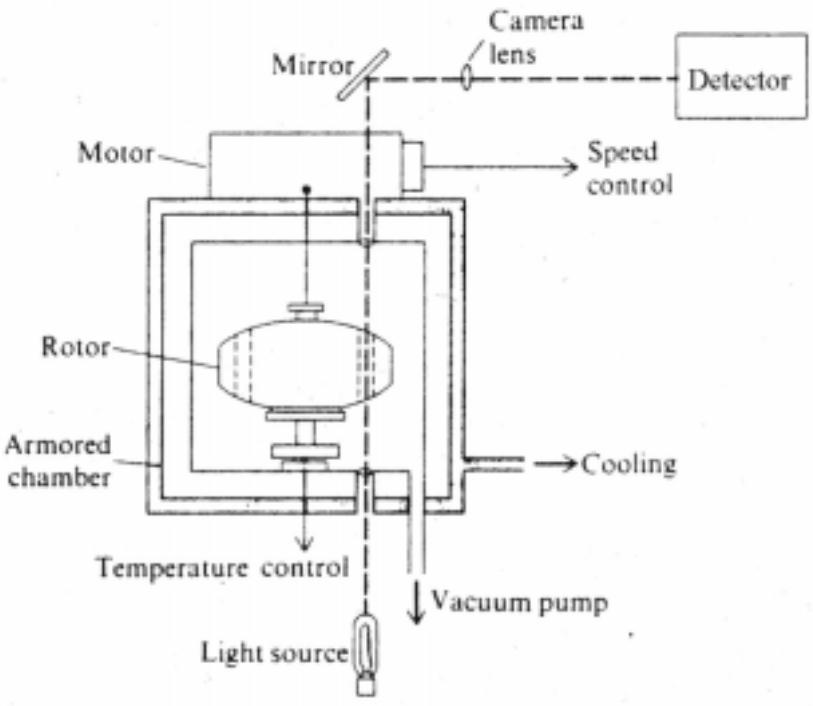


fig. 1 **An analytical ultracentrifuge.** Samples spinning in transparent sector cells within the rotor are monitored online either by UV/VIS spectroscopy or interference spectroscopy (Light source, Detector system).

stochiometry.

Analytical ultracentrifugation experiments were performed on a Beckman XL-A ultracentrifuge in 10 mM Na-phosphate buffer (pH=5.5), 137 mM NaCl. 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 2 h at 24000 rpm (overspeed step), and were subsequently spun at an equilibrium speed of 20000 rpm for 30 – 32 h (equilibrium step). The radial concentration profile of the $d(CG)_3T_4(CG)_3$ hairpin and the Zα protein was measured by UV absorption at six equidistant wavelengths ranging from 255 – 285 nm. The approximate extinction coefficients were calculated from the sequences as described for the DNA [9] and the protein [10].

Data collection and analysis of the four component system, free hairpin, free Zα, complex Zα/hairpin and $(Z\alpha)_2$/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 [11] [12]. 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 an exponential function for each of the four species as described [11] [13]. This global fitting was performed for the various Zα/hairpin ratios of the titration yielding the equilibrium dissociation constant, Kd. The resulting ratios of bound Zα/hairpin yielded a binding curve (Fig. 2B), from which the binding stochiometry was obtained. Since the partial concentrations for the Zα/hairpin and $(Z\alpha)_2$/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 [11] [13].

In order to maintain cysteine 125 of wild-type Zα in the reduced form, the ultracentrifugation runs with wild-type protein were performed under strongly reducing conditions (100 mM β-mercaptoethanol). This was not necessary for the Zα-C125S mutant.

Surface plasmon resonance

Surface plasmon resonance (SPR) spectroscopy is a time-efficient technique to measure both binding constants and binding kinetics of interactions involving biological macromolecules. In a SPR spectrometer, which is more commonly known as a BIAcore instrument, a light beam is directed under a fixed angle onto a very thin golden chip (fig. 2). The intensity of the refracted light, which hits on the detector, is dependent on the refractive index of the solution on the opposite side of the golden chip. On this side, the molecule under investigation is immobilized. If a ligand binds to that molecule the refractive index changes leading to a signal on the detector that is proportional to the total mass bound. By passing a constant flow of buffer with and without ligand over the surface of that chip, binding and dissociation of this ligand can be monitored as a function of time. These data allow the calculation of binding rates and binding constants.

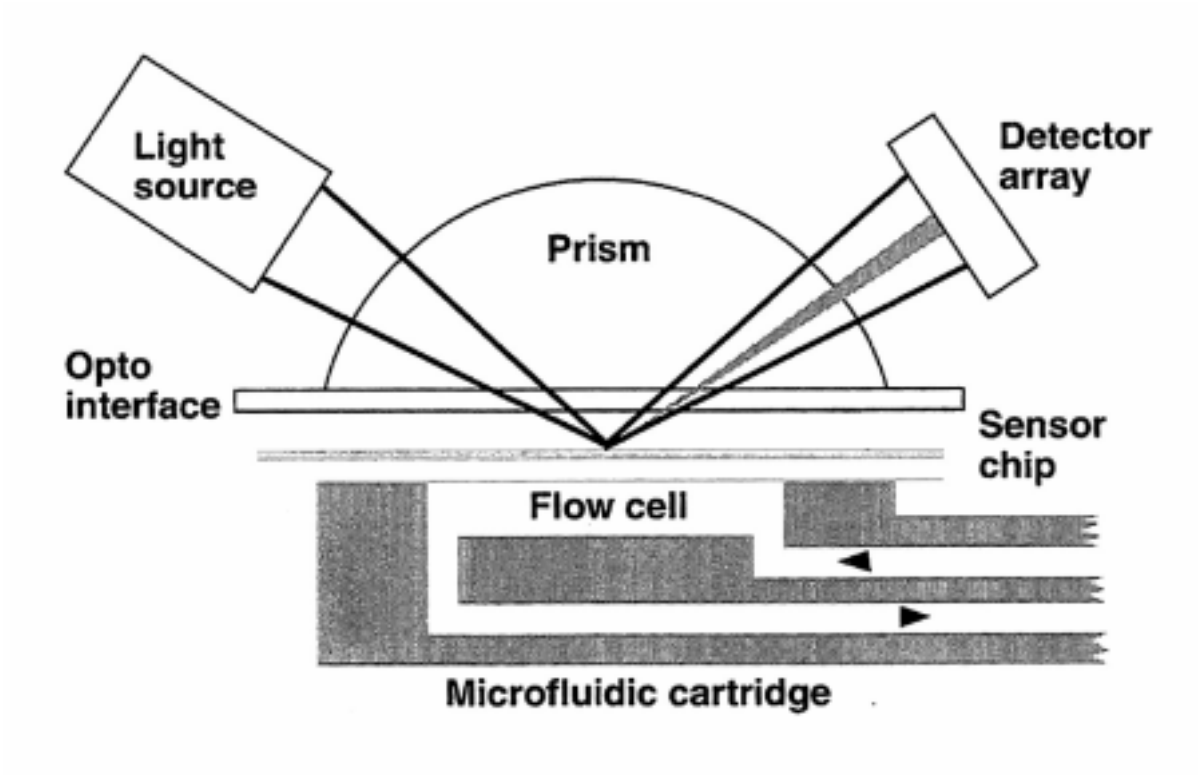


fig. 2 **Application of surface plasmon resonance (SPR) for measuring binding kinetics in solution.** A ligand (in this case DNA), which has been immobilized on the sensor chip, projects into the aqueous solution of the flow cell. An analyte (in this case wild-type and mutant Zα protein) is passed over the chip surface at a constant flow rate allowing the analyte to bind to the immobilized ligand. Bound analyte changes the refractive index leading to signal on the light detector that is proportional to the mass of analyte bound.

The total mass of ligand binding to or dissociating from the immobilized molecule is measured in arbitrary units, designated Response Units (RU). The detection of the change in total mass bound brings about the advantage that labeling of the ligand and knowledge of its physical properties is not required for detection. However, the detection of mass necessitates that the molecular weight of ligands is greater than approximately 1 kD, thereby limiting the range of ligands that can be studied by SPR. In most cases, the ligand can be washed off the chip allowing multiple measurements in a row on the same chip surface. Thus various ligands, such as different mutants of the Zα protein, can be tested under identical conditions in an automated binding assay.

The binding constants of mutant and wild-type Zα were measured on a BIAcore 2000 instrument. 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, 1mM EDTA and 1 mM DTT. The change in surface plasmon resonance was determined at a data collection rate of 1 Hz. 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 does not bind

to a random sequence B-DNA polymer of the same size and immobilized at the same density as the Z-DNA probe [3].

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) to cross-validate the equilibrium dissociation constant (K_d). 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 [14]. Therefore, for calculation of the K_d, steady-state fitting to Langmuir isotherms was considered to be more robust. The protein concentrations used to measure the steady-state response covered an approximately 10-fold concentration range around the K_d of wild-type or mutant protein. Measurement at high flow rates of 50 and 100 µL/min did not improve the quality of the data significantly. Fast binding rates are common for protein/DNA interactions. Furthermore, it is possible that Zα binding is considerably accelerated through 1D diffusion on the polyd(CG) string as compared with a 3D search for free binding sites.

For the K_d measurements of wild-type and C125S mutant protein, the long Zα construct was used which was also utilized for analytical ultracentrifugation and NMR spectroscopy. In contrast, the short Zα construct (residues 122-197) was used for the investigation of Zα mutants from α3 and the C-terminal β-sheet by surface plasmon resonance. This short Zα protein showed a K_d of 70 nM ± 30% which is greater by a factor of 2.3 than the K_d (30 nM) obtained for the long Zα construct (residues 119 – 200). The NMR structure of Zα shows long-range NOEs for residue A198 suggesting that A198 is an integral part of the Zα core fold. Therefore, the C-terminal β-sheet of the short Zα construct, which lacks A198, may be less stable than that of the long Zα construct. This may explain the two-fold reduction in binding affinity because four residues in the C-terminal β-sheet mediate Z-DNA contacts in the crystal structure of the $(Z\alpha)_2$/Z-DNA complex [6].

Circular dichroism spectroscopy

The circular dichroism (CD) titration of 10 µM $d(CG)_3T_4(CG)_3$ hairpin with increasing amounts of Zα protein (fig. 20) was recorded in 10 mM Na-phosphate buffer (pH 5), 75 mM NaF, 0.1 mM NaN_3 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. The characteristic CD spectrum of d(CG) oligomers in the Z-conformation at high ionic strength (4 M NaCl) is shown elsewhere [15].

The CD spectra of mutant and wild-type Zα alone (data not shown) were recorded at 5 µM in 10 mM Na-phosphate (pH 7.2), 137 mM NaCl and H_2O at 25 ^{0}C on an Aviv 60DS CD spectrometer using a cuvette with a 5 mm pathlength.

The CD spectra showing the B- to Z-DNA transition induced by the mutants Y177A and K181A, and by Zα wild-type (fig. 26), were acquired at 30° C in 50 mM Tris/HCl (pH 7.4), 25 mM NaCl and 0.1 mM EDTA on the Aviv CD spectrometer using the 5 mm cuvette. For CD titrations, polyd(CG) (50 µM bp concentration) was titrated with mutant and wild-type Zα in 6:1, 3:1 and 1:1 (mole bp) : (mole Zα) ratios. The stochiometric ratio is 3:1 (mole bp) : (mole Zα) reflecting that two Zα domains bind to six d(CG) basepairs.

Bandshift and Southwestern assay

Bandshift assays, which are also called gelshift and electromobility shift (EMSA) assays, are widely used to measure the binding of a protein to nucleic acids or to other proteins. For bandshift assays the binding partners are pre-incubated for a few minutes and are loaded on a native polyacrylamide gel, in which the bound and free species are separated by electrophoresis. For the study of protein/DNA interactions, the DNA is commonly radioactively labeled with ^{32}P allowing both qualitative detection by autoradiography films and quantitative detection by phosphor-imaging instruments.

The study of protein/Z-DNA interactions by bandshift assays requires a special Z-DNA probe that maintains the Z-DNA conformation under near physiological conditions. This has been achieved by incorporating 5-bromo-desoxycytidine triphosphate and (α-^{32}P)-labeled dGTP into a $d(CG)_n$ polymer using the Klenow fragment of *E. coli* [16]. The bromination ensures that the DNA probe adopts the Z-DNA conformation at Mg^{2+} concentrations of 10 mM. This $(5\text{-Br-dCdG})_{33}$ Z-DNA probe was used for both bandshift and Southwestern assays.

Mutant and wild-type Zα proteins were tested for specific binding to Z-DNA by bandshift assays using non-denaturing 6 % polyacryamide gel electrophoresis [16]. For each lane, Zα protein was incubated with 100 pg Z-DNA probe (~ 40000 cpm) for 10 min in a 20 µL reaction volume containing 1 µg of sonicated salmon sperm DNA, 10 mM $MgCl_2$, 25 mM NaCl and 25 mM Tris/HCl (pH 7.4). After addition of 2 µL loading buffer (Bromphenolblue in a saturated Ficoll solution), the samples were loaded on a 20 cm gel and separated by electrophoresis at 250 V. The bands were visualized by autoradiography using Reflection NEF 496 film (Dupont).

The Southwestern asssay combines a Western blot with Z-DNA probe detection (the „Southern“ part of the assay) [17]. Mutant GST-fusion proteins were separated by standard 15 % SDS-polyacrylamide gel electrophoresis and blotted onto a 0.2 µm reinforced nitrocellulose membrane (BioRad) in Tris/glycine buffer (25 mM Tris, 192 mM glycine), 20 % methanol at 4° C using an electric field of 200 V (Mini-Protean II System, BioRad). Colored molecular mass markers (BioRad) were used as standards. The membrane was blocked twice for 30 min each in approximately 15 mL PBS, 0.5 % Igepal (Sigma), 5 mM DTT and 1 % BSA. After washing twice with 15 mL PBS, 0.5 % Igepal for 10 min each, approximately 5 ng Z-DNA probe (~ $2*10^6$ cpm), 100 µg sonicated salmon sperm DNA and 16 mM $MgCl_2$ were briefly pre-incubated in a volume of 250 µL, diluted with 5 mL PBS, 0.5 % Igepal and incubated with the membrane for 45 – 90 min. After washing three times with PBS, 0.5 % Igepal, the membrane was dried and exposed to an autoradiography film (Reflection NEF 496, Dupont).

The amount of protein blotted onto the membrane was determined by Western blot. The wet membrane was incubated with 5 µL anti-GST-antibody (Pharmacia) in 5 mL PBS, 0.5 % Igepal for 45 min. After three washing steps with 5 mL PBS, 0.5 % Igepal, the membrane was incubated with 1 µL of the secondary antibody anti-(GST-antibody)-alkalic-phosphatase for 45 min. After washing twice with 10 mL PBS, 0.5 % Igepal, the membrane was incubated with 5 mL alkalic phosphatase substrate mix (BCIP/NBT, Kirkegaard & Perry) until clear intense bands emerged (5 – 10 min).

GST-fusion mutant proteins showed a varying extent of C-terminal proteolytic degradation *in vivo* [5]. By using Z-DNA probe detection and Western blot in conjunction on the same membrane, the proteolytic fragments could be separated by molecular weight and individually tested for binding to Z-DNA. Since only full length protein bound to Z-DNA, the amount of full length protein used for Southwestern assays was adjusted to give Western blot bands of equal intensity.

NMR spectroscopy

NMR experiments were carried out on 1.8 - 2 mM samples in 10 mM Na-phosphate buffer (pH 5), 137 mM NaCl and 0.1 mM NaN_3 (10% D_2O) at 25° C on 500, 600 and 750 MHz NMR spectrometers (self-built instruments at Francis Bitter Magnet Laboratory, MIT, Cambridge) and 600 and 750 MHz NMR spectrometers (Bruker instruments at FMP, Berlin). ^{1}H, ^{15}N and ^{13}C resonance assignments were obtained from the following 3D heteronuclear correlation NMR experiments [18]: ^{15}N-HSQC-TOCSY, CBCA(CO)NH, CBCANH, HBHA(CO)NH, H(CCO)NH, HCCH-COSY, HCCH-TOCSY and HNHA.

$^{3}J_{HNHA}$ coupling constants were measured from a 3D HNHA spectrum (ξ=14ms) using integrated diagonal and cross peaks. Apparent coupling constants were multiplied by a factor of 1.11 to correct for Hα spin flips [19].

Interproton distance restraints were derived from 3D ^{15}N-HSQC-NOESY (70, 150 and 250 ms mixing times), 3D ^{13}C-HSQC-NOESY (40, 70 and 100 ms mixing times for aliphatic region; 35 and 70 ms mixing times for aromatic region) and 2D NOESY in D_2O and H_2O (20, 40, 80 and 160 ms mixing times). Spectra were processed with NMRpipe [20] or XwinNMR (Bruker GmbH) and analyzed with FELIX'97 (MSI Inc.). 2D strips from 3D ^{15}N-HSQC-NOESY experiments were extracted and sequentially aligned using the programs Pipp and Plotseq [21]. Spectra were indirectly referenced through the magnetogyric ratios by external calibration on DSS [22].

Interaction mapping

For interaction mapping, 2D ^{15}N-HSQC spectra were recorded on free Zα and $(Z\alpha)_2$/Z-DNA complex in the same buffer as described above at 25 °C. The sample of the complex was prepared by titrating Zα into $d(CG)_3T_4(CG)_3$ DNA at diluted concentrations, and concentrated by ultrafiltration using a stir cell (Amicon, Millipore Corp.). Free $d(CG)_3T_4(CG)_3$ forms a hairpin with a 6 basepair $d(CG)_3$ stem in the B-DNA conformation in solution [4]. The saturation level of 2 Zα to 1 hairpin was monitored by 1D ^{1}H-NMR on well-resolved resonances. Spectra were processed and analyzed as described above.

Structure calculation

NOE restraints derived from all four types of NOESY experiments were analyzed in the conventional manner using a lower boundary of 1.8 Å and an upper boundary of 6 Å and 7 Å for 3D and 2D NOESYs, respectively. Structures were calculated by dynamic simulated annealing from random coordinates [23] at 2000 K using X-PLOR 3.1 [24] and floating stereospecific assignment [25-28]. Intraresidual NOEs were omitted. Force constants for NOE

and phi angle restraints were 50 kcal mol^{-1} $Å^{-2}$ and 200 kcal mol^{-1} rad^{-2}, respectively. Force constants for bond lengths, bond angles and improper angles were 1000 kcal mol^{-1} $Å^{-2}$, 500 kcal mol^{-1} rad^{-2} and 500 kcal mol^{-1} rad^{-2}, respectively.

The atomic coordinates of the 15 lowest energy structures of a total of 200 have been deposited in the Protein Data Bank (PDB) and can be retrieved via the internet at: www.rcsb.org with the PDB ID code: 1qgp.