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Rationally designed helix–turn–helix proteins and their conformational changes upon DNA binding

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Circular dichroism and electrophoretic mobility shift studies were performed to confirm that dimerized N-terminal domains of bacterial repressors containing helix–turn–helix motifs are capable of high-affinity and specific DNA recognition as opposed to the monomeric N-terminal domains. Specific, high-affinity DNA binding proteins were designed and produced in which two copies of the N-terminal 1–62 domain of the bacteriophage 434 repressor are connected either in a dyad-symmetric fashion, with a synthetic linker attached to the C-termini, or as direct sequence repeats. Both molecules bound to their presumptive cognate nearly as tightly as does the natural (full-length and non-covalently dimerized) 434 repressor, showing that covalent dimerization can be used to greatly enhance the binding activity of individual protein segments. Circular dichroism spectroscopy showed a pronounced increase in the α-helix content when these new proteins interacted with their cognate DNA and a similar, although 30% lower, increase was also seen upon their interaction with non-cognate DNA. These results imply that a gradual conformational change may occur when helix–turn–helix motifs bind to DNA, and that a scanning mechanism is just as plausible for this motif class as that which is proposed for the more flexible basic-leucine zipper and basic-helix–loop–helix motifs.

Key words: bacterial repressors/circular dichroism/conformational changes/DNA-binding proteins/helix–turn–helix proteins

Introduction

DNA-binding proteins undergo various conformational changes when binding to their target sites in DNA (for a review, see Spolar and Record, 1994). It is reasonable to suppose that, before reaching a specific recognition site, the protein would first interact with other, non-specific, DNA segments. However, pronounced conformational changes due to non-specific interactions could not be shown convincingly by either spectroscopic (O'Neil *et al.*, 1990) or thermodynamic methods (cf. Spolar and Record, 1994). As experimental evidence for this phenomenon is extrapolated from peptide models devoid of specific DNA-binding activity (Johnson *et al.*, 1994), the existence of a conformational change upon non-specific DNA binding is considered an open question (von Hippel, 1994).

DNA-binding protein domains frequently contain helical segments that interact specifically with DNA. Eventual changes in the conformation of the DNA-binding helices can thus be conveniently monitored by circular dichroism difference spectroscopy, provided that the binding is detectable at sufficiently low DNA–protein ratios. This is in fact the case for segments of the basic-leucine zipper [bZIP] (Vinson *et al.*, 1989; O'Neil *et al.*, 1990, 1991; Talanian *et al.*, 1990; Weiss *et al.*, 1990; Saudek *et al.*, 1991; Anthony-Cahill *et al.*, 1992; Ellenberger *et al.*, 1992; König and Richmond, 1993) and for the basic-helix–loop–helix (bHLH) domains (Anthony-Cahill *et al.*, 1992; Ferré-D'Amaré *et al.*, 1993). The helix–turn–helix (HTH) motif (Harrison and Aggarwal, 1990) seems to be a special case in this respect since it is believed to dock without major conformational change, even to specific target sites (von Hippel and Berg, 1986; Berg and von Hippel, 1988; Spolar and Record, 1994). For example, the N-terminal domain of the phage 434 repressor has almost the same conformation in solution (Neri *et al.*, 1992) as in the DNA complex (Aggarwal *et al.*, 1988). In contrast, NMR spectroscopy of the Trp repressor showed that the recognition helix of the HTH motif undergoes a conformational stabilization upon binding to cognate DNA (Arrowsmith *et al.*, 1990; Youderian and Arvidson, 1994; Zhang *et al.*, 1994). A similar change—an elongation of the recognition helix—was found to take place upon the binding of the NK-2 homeodomain HTH motif to cognate DNA (Tsao *et al.*, 1994). However, conformational changes upon binding to non-cognate DNA have not been reported.

In this paper, we ask whether a helix–turn–helix based DNA-binding molecule which is capable of sequence-specific DNA recognition *in vitro*, changes its conformation when interacting with cognate or non-cognate DNA. As a criterion of specific DNA binding, we used a positive electrophoretic mobility shift assay in the presence of a large (>1000-fold) excess of competitor DNA. Specific DNA binding is an important criterion, in our view, since helical transitions in short, amphiphilic peptides can be relatively easily induced by a variety of agents, and not only by DNA (von Hippel, 1994). Our working hypothesis is that an induced-fit like conformational fluctuation may be a prerequisite for a protein to scan a large number of DNA sites, i.e. it is a necessary step in explaining the speed by which protein ligands reach their target sites. With the present work we sought to prove, on the one hand, that the helix–turn–helix motif is not an exception to this rule, and on the other that the protein conformation appreciably changes upon contact with non-specific DNA.

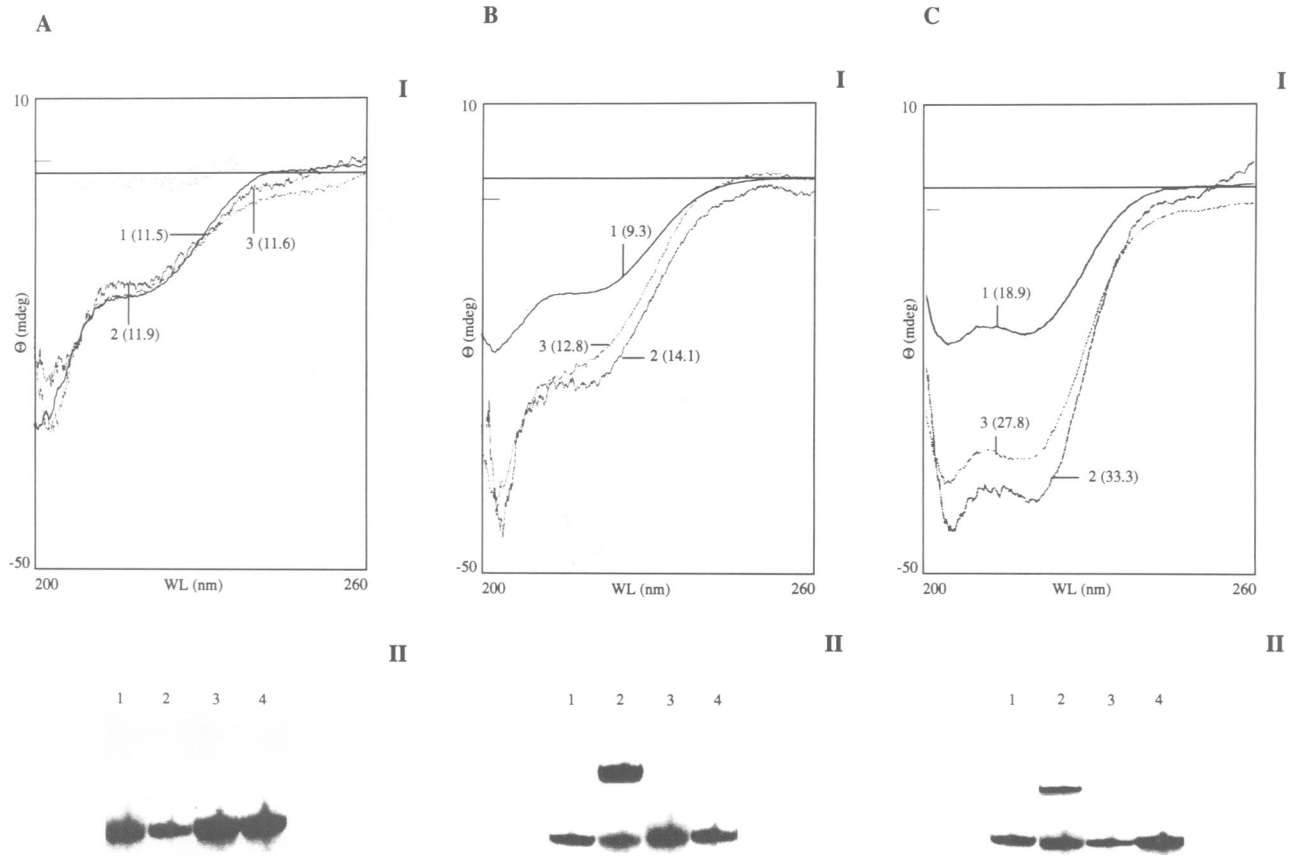

Fig. 1. Induction of the α-helical structure and the specific DNA-binding activity of the peptides studied (sequences shown in Figure 3). (**A**) Nter (the N-terminal 1–63 domain of the phage 434 repressor) (**B**) ChD, a dimeric single-chain repressor with palindromic symmetry (schematic structure Figure 2, sequence and synthesis: Figure 3) (**C**) ReD, a dimeric single-chain repressor molecule with direct repeat symmetry (schematic structure Figure 2, sequence and synthesis Figure 3). I: induction of α-helical structure by cognate and non-cognate DNA as determined by circular dichroism difference spectroscopy (O'Neil *et al.*, 1990; Talanian *et al.*, 1990). Top curves (1) are the spectra of the peptides in the absence of DNA. The bottom curves (2) are the spectra induced by cognate DNA (OR). The centre curves (3) are the spectra induced by non-specific DNA (NS, see sequences in Figure 3). The numbers in parentheses indicate the nominal α-helical content of the respective molecule, calculated according to Wu *et al.* (1984). Spectra were recorded at 24 μM DNA duplex and 24 μM protein dimer (48 μM Nter) concentrations in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.5, at 25°C. II: specific DNA binding as detected by electrophoretic mobility shift assay. Lanes 1, cognate DNA (OR) alone; lanes 2, cognate DNA (OR) + protein (Nter for section A, ChD for section B and ReD for section C); lanes 3, non-cognate DNA (NS) alone; lanes 4, non-cognate DNA (NS) + proteins as listed at lanes 2.

Results

As an experimental model we chose the N-terminal (Nter) domain of the phage 434 repressor (Aggarwal *et al.*, 1988; Neri *et al.*, 1992). This domain can be co-crystallized with cognate DNA, but its DNA-binding affinity is low (Aggarwal *et al.*, 1988). We found that Nter does not bind to the cognate DNA in the presence of competitor DNA (Figure 1AI) and it shows no appreciable increase in its helical content upon addition of *ds* oligonucleotides that contain either cognate or non-cognate sequences (Figure 1AII). This could mean, in principle, that the Nter domain is essentially rigid, as is sometimes supposed (Johnson *et al.*, 1994; Spolar and Record, 1994). On the other hand, the native 434 repressor, which has a high and specific DNA-binding activity, binds to cognate DNA as a dimer, non-covalently connected through a large C-terminal dimerizaton domain (Anderson *et al.*, 1984, Figure 2A). Since structural transitions may not be monitored accurately in the presence of a large dimerization domain, we decided to design molecular probes in which the specificity of the dimeric repressor is mimicked by a covalent linkage. As the resulting molecules consist of a single chain, we call these covalent dimers single-chain repressors. The first covalent dimer, ChD (Figure 2B, sequence shown in Figure 3) has a palindromic (dyadic) symmetry since the two Nter domains are connected through their C-termini with a flexible and symmetric synthetic linker of ~36 Å maximum length. The second single-chain repressor, ReD (Figure 2C, sequence in Figure 3), contains two direct sequence repeats of the Nter sequence connected with a longer linker (~96 Å). This molecule was produced by recombinant DNA methods (A.Simoncsits, F.Chen, S.Wang, P.Percipalle and S.Pongor, manuscript in preparation). Both molecules are capable of specifically binding cognate DNA in the presence of competitor DNA and do not show gel-mobility shift with non-cognate DNA under the same conditions (Figure 1BII and 1CII, respectively). The dissociation constants (K_ds) were estimated to be $5–6\times10^{-9}$ for ReD and $2–3\times10^{-8}$ for ChD, based on the protein concentrations at which 50% binding was detected in electrophoretic mobility shift assays. Under the same conditions, the native repressor had a K_d of $2–4\times10^{-9}$, while the binding of Nter was not measurable (data not shown).

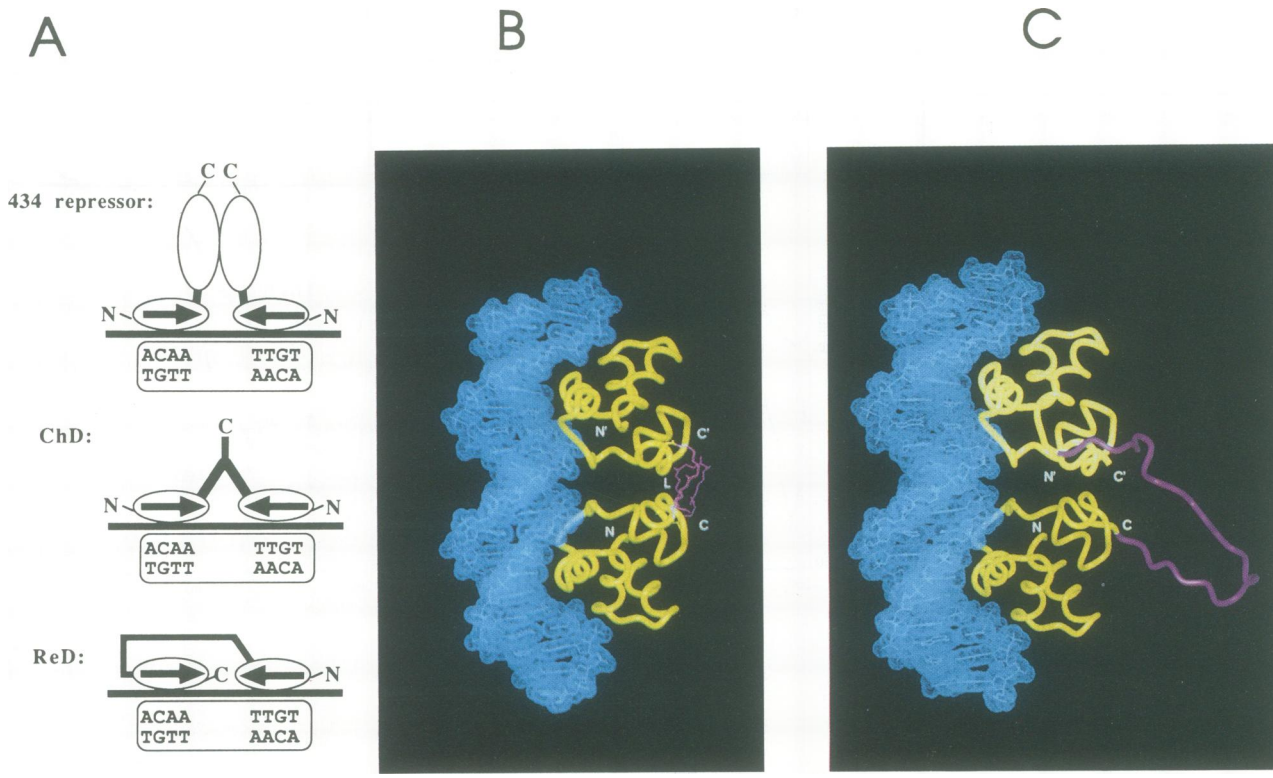

Fig. 2. Schematic structure of the 434 repressor and of the single-chain analogues, ChD and ReD. (**A**) Scheme of binding of the 434 repressor, ChD and ReD to cognate DNA. The arrows denote the N-terminal domain (N to C direction). (**B**) Predicted 3-D structure of the dimeric single-chain repressor ChD. (**C**) Predicted 3-D structure of the dimeric single-chain repressor ReD. The linker in ReD is deemed to be flexible, its position is only symbolically shown in (C).

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                1                   20                  40                  60
                |                   |                   |                   |
Nter:           SISSRVKSKRIQLGLNQAELAQKVGTTQQSIEQLENGKTKRPRFLPELASALGVSVDWLLNGT
                |-------H1-----|--H2--| T|----H3---|---L---|-----H4----|--H5-->

                          cI repressor of 434 phage (1-63)

ChD  [Nter(1-62)-Ahx2-]Lys[Nter(1-62)-Ahx2]-Gly-OH

ReD  Nter-SDSNVRFVGHVEPKGKYPLISMVRAG-Nter-SDSNVR
           |------cI 434 64-89------|

OR     5'-TACAAGAAAGTTTGTTA
          ATGTTCTTTCAAACAAT-5'

NS     5'-AGAGGAGCGCCTGTCATGCA
               CTCGCGGACAGTACGTCGCG-5'
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Fig. 3. Amino acid and nucleotide sequences. Nter, sequence of the 434 phage repressor protein N-terminal domain Nter (Kuziel and Tucker, 1987; Aggarwal *et al.*, 1988); H, helix; T, turn; L, loop regions; ChD, sequence of ChD, the dimeric single-chain repressor produced by chemical synthesis; Ahx, ε-aminocaproic acid; ReD, sequence of ReD, the dimeric single-chain repressor produced by recombinant DNA in *E.coli*. The sequence corresponds to (1–89)(1–69) of the cI repressor of 434 phage. OR, cognate DNA, the O_R1 operator site of the 434 phage; NS, oligonucleotides used as non-specific DNA.

In contrast to Nter, both ChD and ReD show a marked increase in their helical content (signal at 222 nm) upon the addition of cognate DNA (curves 2 in Figure 1BI and 1CI, respectively). A qualitatively very similar conformational change is observed in the presence of non-cognate DNA (curves 3 in Figure 1BI and 1CI); the change induced by non-cognate ds DNA is ~70% (69% for ChD and 73% for ReD) compared with that induced by cognate DNA. Single-stranded DNA molecules (either cognate or non-cognate sequences) failed to induce helicity in either of the peptides (not shown). Cognate and non-cognate DNA seems to increase ordered structure both in ChD and in ReD, even though the original conformations of these two molecules are slightly different; ChD apparently contains more 'random-coil' (200 nm signal) than does ReD. This difference (as well as the higher K_d value of ChD) may be attributed to the fact that ChD has a much shorter linker than ReD; moreover, the relatively harsh synthesis conditions may result in partial denaturation. If we suppose that: (i) all molecules in solution have a

conformation corresponding to the X-ray structure; and (ii) none of the residues in the linkers has helical conformation, then the expected maximal α-helix content of ChD would be 57% while that of ReD would be 51%. The nominal helicity calculated from the CD data is lower than these values (9.3–14.1 for ChD and 18.9–33.3 for ReD). The differences may originate either from the approximate nature of the CD-based helicity calculation (Wu *et al.*, 1981) or, more probably, from the fact that in the solution environment used for CD studies, not all molecules have ordered helical conformations. In fact, adding 10% trifluoroethanol to the medium approximately doubles the α-helix signal both in ChD and in ReD indicating that the peptides can adopt more helical conformations under favourable conditions (Percipalle, 1995).

Discussion

Our results show that: (i) high-affinity DNA-binding molecules can be obtained by combining two HTH-containing DNA-binding domains; and (ii) the single-chain repressors described here change their conformation upon interaction with non-specific as well as with specific DNA sites.

The combination of two HTH-containing domains to give artificial DNA-binding proteins is not new in itself. Several DNA-binding domains were fused with dimerization domains to give molecules that yield DNA-binding dimers. Examples include the lambda repressor head–GCN4 leucine zipper fusions (Hu *et al.*, 1990, 1993), the Lex A repressor head–jun leucine zipper fusions (Schmidt-Dörr *et al.*, 1991), the LexA core–gal4 fusions (Brent and Ptashne, 1985), the 434 repressor N-terminal domain/leucine zipper fusions (Pu and Struhl, 1993) and the lambda repressor N-terminal domain/*rop* fusion (Castagnoli *et al.*, 1994). While all of these fusion proteins show specific DNA binding, they are not especially useful as probes to monitor conformational changes in the recognition helices, since they contain either large, or highly helical dimerization domains that would make CD difference spectroscopy impractical. A further distinctive feature of our constructs, in comparison with earlier fusion proteins, is the fact that we based our design on covalent dimerization ('single-chain architecture') which in itself proved sufficient to reach a DNA binding affinity that is comparable with that of the corresponding full-length repressor molecule.

Fundamentally, the results confirm the principle on which the design of the molecule was based: the tightness and the specificity of the binding critically depend on a dimeric architecture. In fact, both very different covalent dimerization strategies adopted here successfully mimic the non-covalent dimerization of the full-length 434 repressor since they have K_ds that are comparable with that of the natural repressor. The dramatic enhancement of binding strength as compared with that of a monomeric Nter domain may be explained partly by an increase of the binding surface and partly by a decrease in the dissociation rate of the bound protein. A possible, structural explanation of the strong DNA-binding affinity could be that the Nter domains have a structured dimer interface sufficient to bring about dimerization once the two Nter domains are brought together by a suitable linker.

The data indicate that the Nter domain is not rigid, but seems rather to adopt its binding conformation when it comes into contact with DNA. More interestingly, a conformational change is already detectable when the protein comes into contact with non-specific DNA. The difference between 'rigid' (HTH) and 'flexible' (bZIP, bHLH) motifs may, thus, be less dramatic as previously thought (von Hippel and Berg, 1986; Berg and von Hippel, 1987, 1988; Harrison and Aggarwal, 1990; Johnson *et al.*, 1994; Spolar and Record, 1994; von Hippel, 1994) and an induced-fit like model of recognition (Koshland, 1958) also seems to be valid for the HTH-domain. In fact, one may speculate that the flexibility of the Nter domain may be essential for the repressor to scan DNA in search of a specific target site, in the same manner as is proposed for more flexible DNA-binding domains (O'Neil *et al.*, 1990; Johnson *et al.*, 1994). The conformational change induced by non-cognate DNA is qualitatively similar and only somewhat weaker (70%) compared with that caused by cognate DNA. It is tempting to argue that the change is in fact gradual: the protein fluctuates in a larger conformational space when in contact with non-cognate DNA and a final, smaller, set of helical conformations is stabilized only at the target site. It must be emphasized that the present results show the conformational changes taking place within the N-terminal domain but do not prove unequivocally that these take place solely within the DNA-recognition helix. The fact that the Nter domain in solution (Neri *et al.*, 1992) has a conformation almost identical to the Nter domain–O_R1 complex (Aggarwal *et al.*, 1988) does not contradict our findings. CD spectroscopy gives a signal proportional to the number of molecules in the ordered (α-helical) conformation. This signal will be higher if the DNA drives the conformational equilibrium of the protein toward the helical state (which seems to be the case), irrespective of the fact that the helical conformations of the free and complex-bound states are identical or different.

Finally, we mention that a series of dimeric peptides incorporating shorter segments of the Nter domain had been designed and synthesized (Percipalle *et al.*, 1994; Percipalle, 1995), but they failed to show specific DNA binding under the experimental conditions used in this study.

Materials and methods

Computer modelling

The structure of both ChD and ReD were modelled using the Insight/Discover package (Biosym), by manually inserting the linker sequences into the DNA–N-terminal domain complex built using the published 3D co-ordinates of the 434 repressor N-terminal domain–DNA complex (Aggarwal *et al.*, 1988), and then subjecting the linker structures first to a 100 ps Molecular Dynamics at 300 K with a distance-dependent dielectric constant and then to 200 cycles steepest descent energy minimization. The known part of the structure was kept fixed during the entire simulation.

Peptide and oligonucleotide synthesis

Nter and ChD (sequences shown in Figure 3) were prepared via automated continuous-flow solid-phase peptide synthesis, using the Fmoc chemistry, as described (Percipalle *et al.*, 1995). ChD is a branched synthetic peptide in which the C-terminal residue of the Nter sequence is joined to both amino groups of a central lysine residue linked to the resin via a glycine. The peptides were purified by reverse-phase HPLC to electrophoretic homogeneity; the yields were 25% for Nter and 1% for ChD.

The oligonucleotides OR and NS (sequences shown in Figure 3) were prepared by the ICGEB oligonucleotide synthesis service.

Recombinant DNA methods

ReD was produced by recombinant DNA techniques and was expressed in *Escherichia coli* cells. The plasmid was constructed from λgt10 template (Huynham *et al.*, 1984; Kuziel and Tucker, 1987), by PCR amplification of DNA fragments corresponding to amino acids 1–89 (as a *Rca*I–*Bam*HI fragment) and to amino acids 1–69 (a *Bam*HI–*Hin*dIII fragment). Restriction sites to obtain these fragments were included into the PCR primers. The fragments were cloned consecutively between *Nco*I and *Bam*HI sites and between *Bam*HI and *Hin*dIII sites of *E.coli* expression vectors pRIZ′ (Simoncsits *et al.*, 1994), and a pRSET5d (Schoepfer, 1993) derivative lacking the second *Nco*I site in the cloning region of pRSET5d (A.Simoncsits, unpublished results), resulting in pRIZ′RR69 and pRSETRR69, respectively (where RR69 represents the region coding for ReD). High-level expression of ReD was achieved using the pRSET5dRR69 in BL21(DE3)[pLysS] *E.coli* (Novagen). Purification to >95% homogeneity was achieved using cell lysis by the freeze–thaw method (Studier *et al.*, 1990) followed by batch adsorption to SP–Sepharose (Pharmacia) and HPLC on a Shodex SP 825 cation exchange column.

Circular dichroism spectroscopy

Spectra were recorded at 24 μM DNA duplex and 24 μM protein dimer (48 μM Nter) concentrations in 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.5, at 25°C. Data were collected on a Jasco J-600 spectrometer (Jasco, Inc., Easton, MD, USA) and were smoothed using software provided by Jasco. The nominal α-helix content was calculated according to Wu *et al.* (1981). Difference spectra were calculated as described by O'Neil *et al.* (1990) and by Talanian *et al.* (1990). The spectrum of the peptide–DNA complexes did not show any change in the 250–400 nm range as compared with free DNA that would indicate a major conformational change in DNA (O'Neil *et al.*, 1990).

Electrophoretic mobility shift assays

Binding experiments were performed in 20 μl buffer [50 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 20 mM HEPES, pH 7.9, and 1 μg poly(dI–dC)] containing 20 nM [^{32}P]phosphate DNA-probe and protein (19 μM Nter or 1.6 μM ChD or 1.2 μM ReD), at 4°C for 40 min. Electrophoresis was performed on 8% polyacrylamide gel using Tris-borate buffer at 4°C.

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