

Mutations in the Glucocorticoid Receptor DNA-binding Domain Mimic an Allosteric Effect of DNA

M. A. A. van Tilborg¹, J. A. Lefstin², M. Kruiskamp¹, J.-M. Teuben¹
R. Boelens¹, K. R. Yamamoto² and R. Kaptein^{1*}

¹*Bijvoet Center for Biomolecular Research
Padualaan 8, NL3584CH
Utrecht, The Netherlands*

²*Department of Cellular and Molecular Pharmacology
University of California, San Francisco, CA 94143-0450
USA*

Two previously isolated mutations in the glucocorticoid receptor DNA-binding domain (DBD), S459A and P493R, have been postulated to mimic DNA-induced conformational changes in the glucocorticoid receptor DBD, thereby constitutively triggering an allosteric mechanism in which binding of specific DNA normally induces the exposure of otherwise silent glucocorticoid receptor transcriptional activation surfaces. Here we report the three-dimensional structure of the free S459A and P493R mutant DBDs as determined by NMR spectroscopy. The free S459A and P493R structures both display the conformational changes in the DBD dimerization interface that are characteristic of the DNA-bound wild-type DBD, confirming that these mutations mimic an allosteric effect of DNA. A transition between two packing arrangements of the DBD hydrophobic core provides a mechanism for long-range transmission of conformational changes, induced either by the mutations or by DNA binding, to protein-protein contact surfaces.

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Keywords: glucocorticoid receptor; DNA-binding domain; mutant; allosteric

*Corresponding author

Introduction

The expression of genetic information is governed largely by sequence-specific DNA-binding proteins that activate or repress transcription by RNA polymerase. Although these proteins commonly contain discrete DNA-binding and transcriptional regulatory domains that can be separated artificially, experimental evidence suggests that in some regulatory proteins these domains communicate with each other to operate as integrated units (Lefstin & Yamamoto, 1998).

Examples of such proteins are found in the intracellular receptors, an extensive superfamily of transcriptional regulatory proteins that is represented in all metazoans. Members of this superfamily are characterized by a conserved DNA-binding domain (DBD) with two zinc ions each coordinated by four cysteine residues. Although some intra-

cellular receptor superfamily members bind DNA as monomers, most known members bind to DNA sites as homodimers, heterodimers with another superfamily member, or in conjunction with unrelated DNA-binding proteins.

One member of this family is the vertebrate glucocorticoid receptor (GR). Among the first eukaryotic transcriptional regulatory proteins to be isolated, the GR resides in the cell cytoplasm in the absence of hormone. Upon the binding of corticosteroid hormones to the carboxy-terminal ligand-binding domain, the GR translocates from the cytoplasm to the nucleus and binds to specific DNA sites termed glucocorticoid response elements, or GREs.

Simple GREs, at which the GR activates transcription, are palindromic repeats of sequences similar to AGAACA, separated by three base-pairs; the GR binds to simple GREs as a homodimer. The isolated GR DBD is monomeric in solution; hence, binding to a simple GRE is associated with a monomer-dimer transition of the DBD. Activation by GR at simple GREs depends in part on a strong transcriptional activation domain contained in the GR amino terminus.

The GR also regulates transcription at complex GREs. Complex GREs are less well-characterized than simple GREs, but include composite GREs, at

Abbreviations used: GR, glucocorticoid receptor; DBD, DNA-binding domain; GRE, glucocorticoid response elements; WT, wild-type; RXR, retinoid X receptor; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy.

E-mail address of the corresponding author: kaptein@nmr.chem.uu.nl

which GR binds to DNA in conjunction with other proteins, and tethering GREs, at which GR recognizes DNA only indirectly, by contacting other DNA-bound proteins. Depending on the arrangement of binding sites and identity of other proteins bound at the complex GRE, the GR may activate or repress transcription. The mechanism of repression by GR is not well understood, but genetic and biochemical data have been interpreted to suggest that dimeric complexes of GR activate transcription, while monomeric or unpaired GRs are repressive (Lefstin & Yamamoto, 1998).

The three-dimensional structure of a rat GR DBD fragment (residues 440 to 525) has been determined as a free monomer by NMR spectroscopy (Härd *et al.*, 1990a,b) and as a dimer bound to a simple GRE by X-ray crystallography (Luisi *et al.*, 1991). Although the major secondary structure elements and overall protein fold were similar between the two structures, comparison of the free and bound forms implied that two conformational changes in the second zinc module accompany DBD binding to a GRE. Residues 476 to 491 were ill-defined in the initial free monomer structure (Härd *et al.*, 1990b; van Tilborg *et al.*, 1995). In the GRE-bound dimer, however, residues in each DBD molecule between Cys476 and Cys482 formed part of a well-defined dimer interface between the two DBD molecules. Mutational analysis of this segment, known as the D- or dimerization-loop, indicated that these intermolecular contacts were important for dimerization of the DBD (Dahlman-Wright *et al.*, 1991).

In addition, residues Lys484-Asn491 in the GRE-DBD complex formed a short distorted helical structure not observed in the free DBD. Hence, it was proposed that this helix and the D-loop were structured properly only upon DNA binding. However, a second NMR determination, using a slightly smaller GR DBD fragment than was used in the initial structural studies (439 to 520; Baumann *et al.*, 1993), showed a somewhat more well-defined second zinc finger, including the distorted helix (Lys484-Asn491) and a better defined D-loop (Cys476-Cys484), albeit in a different conformation as compared to the bound X-ray structure.

Lefstin *et al.* (1994) described two mutations in the GR DBD (Figure 1) which implicated these DNA-induced conformational changes in allosteric control of the GR's transcriptional activation functions. Displaying identical phenotypes, these mutations, S459A and P493R, were each single amino acid substitutions originating from random mutagenesis of the DBD (Godowski *et al.*, 1988; Thomas, 1992). When expressed at low levels, both mutant receptors activated transcription as efficiently as the wild-type receptor at GRE-linked test promoters in yeast or mammalian cells. If the mutant receptors were overexpressed, however, their ability to activate transcription at GREs decreased, relative to wild-type. Moreover, transcriptional activation was also attenuated at certain

natural promoters that lacked GREs altogether. For example, transcriptional activation at the yeast GAL1 locus, but not the CUP1 locus, was severely decreased when the mutant receptors were overexpressed and stimulated by hormone. It is apparent that as a result of this transcriptional interference, overexpression of the mutant receptors was lethal to yeast cells in the presence of hormone.

Although the S459A and P493R mutations reside in the DNA-binding domain of the receptor, expression of the mutant DBDs alone was insufficient to cause transcriptional interference or lethality in yeast. Only receptor derivatives that contained a strong transcriptional activation domain, such as that found in the receptor's amino-terminal segment, displayed these phenotypes. Indeed, substitution of the GR amino-terminal activation domain with a heterologous transcriptional activation domain, such as that of the herpesvirus VP16 protein, yielded transcriptional interference and lethality when the activation domain was coupled to either mutant DBD, but not when linked to the wild-type DBD.

The requirement for a transcriptional activation domain suggested that the mutant phenotypes reflected "squelching", i.e. the titration of a limiting target factor by the mutant receptors. Activation domains may function by binding particular target proteins and recruiting them to a promoter at which the activation domain is localized. If the target protein is in limited supply, overexpression of a functional activation surface in *trans* may impede transcriptional activation at a particular locus by sequestering a target protein required for activation at that promoter (Gill & Ptashne, 1988).

As the wild-type GR activates transcription at simple GREs, its transcriptional activation surfaces must be "exposed" when the receptor is specifically bound to a simple GRE. However, the phenotypes of the S459A and P493R mutants implied that they somehow expose transcriptional activation surfaces under conditions where most wild-type receptors in the cell do not, since both wild-type and mutant receptors bear the same activation domain. Lefstin *et al.* (1994) therefore proposed that the GR could exist in two states within the cell, depending on DNA occupancy: an "inactive" state when not bound to a simple GRE, and an "active" form when bound to a simple GRE. Conformational changes in the GR DBD induced by GRE-binding switch the GR between these states, leading to functional exposure of otherwise quiescent transcriptional activation surfaces, i.e. specific DNA acts as an allosteric effector of the receptor's transcriptional activation functions. According to this model, the S459A and P493R mutations in the GR DBD mimic the allosteric effect of DNA, causing the DBD to adopt the GRE-bound conformation even in the absence of specific DNA. As a result, the GR transcriptional activation surfaces are constitutively exposed, whereas in the wild-type protein they are exposed only upon DNA-binding. This constitutive exposure of activation

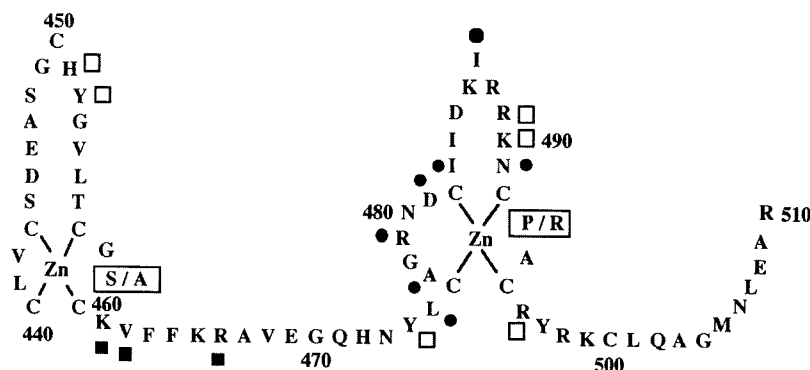


Figure 1. Sequence and zinc-coordination of the rat glucocorticoid receptor DNA-binding domain. (Cys440-Arg510). The S459A and P493R mutations are shown boxed. Residues which in the complex interact with DNA bases (filled squares) and the phosphate backbone (open squares), and which form the dimer interface (filled circles) are also indicated.

surfaces sequesters transcriptional target factors, leading to transcriptional interference and lethality when the mutant receptors are overexpressed.

This model predicted that the free S459A and P493R DBDs would display one or both of the conformational differences observed in the wild-type protein between the free DBD monomer and dimeric DBD-GRE complex: reorientation of the D-loop and formation of the distorted helix. To test this prediction, we determined by NMR spectroscopy the three-dimensional structures of the free wild-type, S459A, and P493R DBDs and compared them to the structure of the DNA-bound wild-type protein.

Results and Discussion

Structure determination

NMR spectra of the wild-type (WT), S459A and P493R DBDs were recorded using a fragment of the rat GR protein (residues Cys440-Lys513). This fragment is slightly shorter than the fragments previously studied by Härd *et al.* (1990b), Luisi *et al.* (1991) and van Tilborg *et al.* (1995) (440-525) as well as Baumann *et al.* (1993) (439-520). Assignment of the NMR spectra of all three receptors was based on previous work of Härd *et al.* (1990a) and van Tilborg *et al.* (1995). The assignment resulted in a set of structural constraints, which were used to calculate an ensemble of 25 structures for each protein. From the ensembles, 22, 23 and 21 structures of the WT, S459A and P493R fragments, respectively, were selected based on low energy and few restraint violations. Table 1 summarizes the experimentally derived constraints and structural statistics of the selected structures.

The calculated ensembles are shown in Figure 2. The ensemble structures are well converged, with the exception of the C-terminal segment which had no long-range NOEs and remains ill-defined. The ϕ and ψ dihedral angle order parameters indicate an overall well-defined backbone (S^2 around 1) with the exception of the tip of the first zinc finger and the linker between the two zinc coordinating domains (Figure 3). The configurations of the first and second zinc finger are the same as in the struc-

tures of the WT GR DBD of Baumann *et al.* (1993) and van Tilborg *et al.* (1995), with S and R chirality for the first and second finger, respectively.

All of the structures contain a distorted helical region (Lys486-Asn491), similar to that seen in the GR-DNA complex (Luisi *et al.*, 1991) and a structure of the free 439-520 fragment (Baumann *et al.*, 1993). This region was disordered in previous determinations of the free 440-525 fragment structure (Härd *et al.*, 1990b; van Tilborg *et al.*, 1995). Nonetheless, it is clearly defined in the 440-513 spectra by several helical NOEs absent from the 440-525 spectrum (see Figure 4). Since the 440-525 and 440-513 spectra were recorded under the same experimental conditions, perhaps C-terminal truncation of the DNA-binding domain fragment somehow favors the establishment of the distorted helix, although the helix is quite distant from the C terminus. This explanation is consistent with the detection of these helical NOEs in the spectrum of the 439-520 DNA-binding domain fragment (Baumann *et al.*, 1993).

Backbone comparison

Figure 5(a) compares the backbone of a representative WT free DBD structure with the previously determined structure of WT DBD bound to specific DNA (Luisi *et al.*, 1991). The free and bound structures are quite similar, except in the vicinity of the D-loop (residues Cys476-Cys482). As previously noted (Luisi *et al.*, 1991), the D-loop in the DNA-bound structure is rotated relative to the free structure by approximately 90° . This conformational change positions residues in the D-loop to make protein-protein contacts with a second DBD molecule on the palindromic GRE. These contacts are important for cooperative DNA binding (Dahlman-Wright *et al.*, 1991) and for transcriptional activation at simple GREs (Yamamoto *et al.*, 1992; Heck *et al.*, 1994).

It is remarkable that free S459A and P493R maintain the D-loop and adjoining regions in a conformation nearly identical to that found in the WT DNA-bound receptor (Figure 5(b)). New long-range NOEs not found in the WT free DBD spectrum (Figure 4) clearly define this altered confor-

Table 1. Summary of constraints used as an input for structure calculation and stereochemical quality of the ensemble of GR DBD structures

Parameter	wt DBD 22 struct.	S459A 23 struct.	P493R 21 struct.
A. NOE values			
Intraresidue	261	269	250
Sequential	350	341	347
Medium range	220	214	219
Long range	322	316	310
Total	1153	1140	1126
ϕ -angle (deg.)	38	42	40
χ 1-angle (deg.)	15	22	20
Number of distance violations >0.5 Å	3	3	1
Maximum distance constraint violation (Å)	0.60	0.57	0.55
B. % of residues^{a,b} with ϕ/ψ in:			
Most favored regions	63.9	61.8	61.5
Additional allowed regions	30.2	31.7	35.0
Generously allowed regions	3.9	2.0	1.9
Disallowed regions	2.0	4.5	1.6
RMSD C440-R510			
Backbone	0.59(±0.07)	0.48(±0.07)	0.48(±0.05)
All atoms	1.14(±0.10)	1.03(±0.10)	1.04(±0.09)
Second domain Q471-R510			
Backbone	0.67(±0.10)	0.52(±0.09)	0.54(±0.07)
All atoms	1.31(±0.12)	1.16(±0.13)	1.16(±0.13)
D-loop C476-C482			
Backbone	0.37(±0.12)	1.16(±0.13)	1.16(±0.13)
All atoms	0.89(±0.19)	1.16(±0.16)	0.97(±0.25)

^a Calculated for the final set of structures with the program PROCHECK (Morris *et al.*, 1992).

^b Residues excluding glycine and proline.

mation in the S459A and P493R spectra. All ensemble structures of S459A and P493R display the altered conformation; although this region is slightly less well-defined in the WT ensemble, in no instance does the WT D-loop approach the orientation found in the mutants and in the DNA-bound structure (Figure 2). A full list of the differences in NOEs observed for the WT and two GR-DBD mutants is provided in Tables I and II of the Supplementary Material.

By adopting the conformation characteristic of the DNA-bound receptor, the D-loop reorientation observed in the free S459A and P493R structures demonstrates unequivocally that these mutants do in fact mimic an allosteric effect of DNA, as predicted from their genetic and biochemical behavior (Lefstin *et al.*, 1994). What is not apparent from the protein backbones is how the S459A and P493R substitutions induce D-loop reorientation, since the backbone shows little perturbation at either residue position. This question is particularly acute for the S459A mutation, which removes a single oxygen atom over 20 Å distant from the D-loop. Understanding the mechanism of conformational change requires detailed examination of the aliphatic side-chains composing the hydrophobic core between the DNA recognition helix and the dimerization interface.

Hydrophobic core reorganization

Figure 6 compares among the structures the arrangement of the DBD's small hydrophobic core,

which comprises a shell of aliphatic side-chains, including Arg496 (red) and Pro493 (yellow), surrounding the aromatic residues Phe463 (green) and Tyr474 (green). This core is exposed along the DBD's DNA-contact surface, and several of the side-chains (Tyr474, Arg489, Lys490 and Arg496) make phosphate contacts with the DNA backbone. As is apparent in Figure 6, the reorientation of the D-loop seen in S459A, P493R and the DNA-bound structure is in each case accompanied by a reconfiguration of the hydrophobic core. The inner aromatic residues Phe463 and Tyr474, somewhat askew in the free WT structure, now assume a parallel arrangement. In S459A and bound WT, Pro493, maintaining its close contact with the aromatic residues, is displaced towards the D-loop, while its pyrrolidine ring flips its orientation and pucker. In P493R, the rigid proline-ring structure has been replaced by a flexible arginine side-chain, but the base of the chain follows a conformation similar to the pyrrolidine ring in S459A and the bound WT, and packs against the aromatic residues as well. His472, which is considerably disordered in the free WT DBD (Hård *et al.*, 1990b; Baumann *et al.*, 1993; van Tilborg *et al.*, 1995), consistently packs against Tyr474 in the S459A, P493R, and specifically bound WT structures. Both residues are indicated in green in Figure 6.

Comparing the structure of the free WT (Figure 6(a)) and S459A (Figure 6(c)) reveals a triggering role for the conserved residue Arg496 in reorganization of the hydrophobic core. The aliphatic portion of the Arg496 side-chain contacts

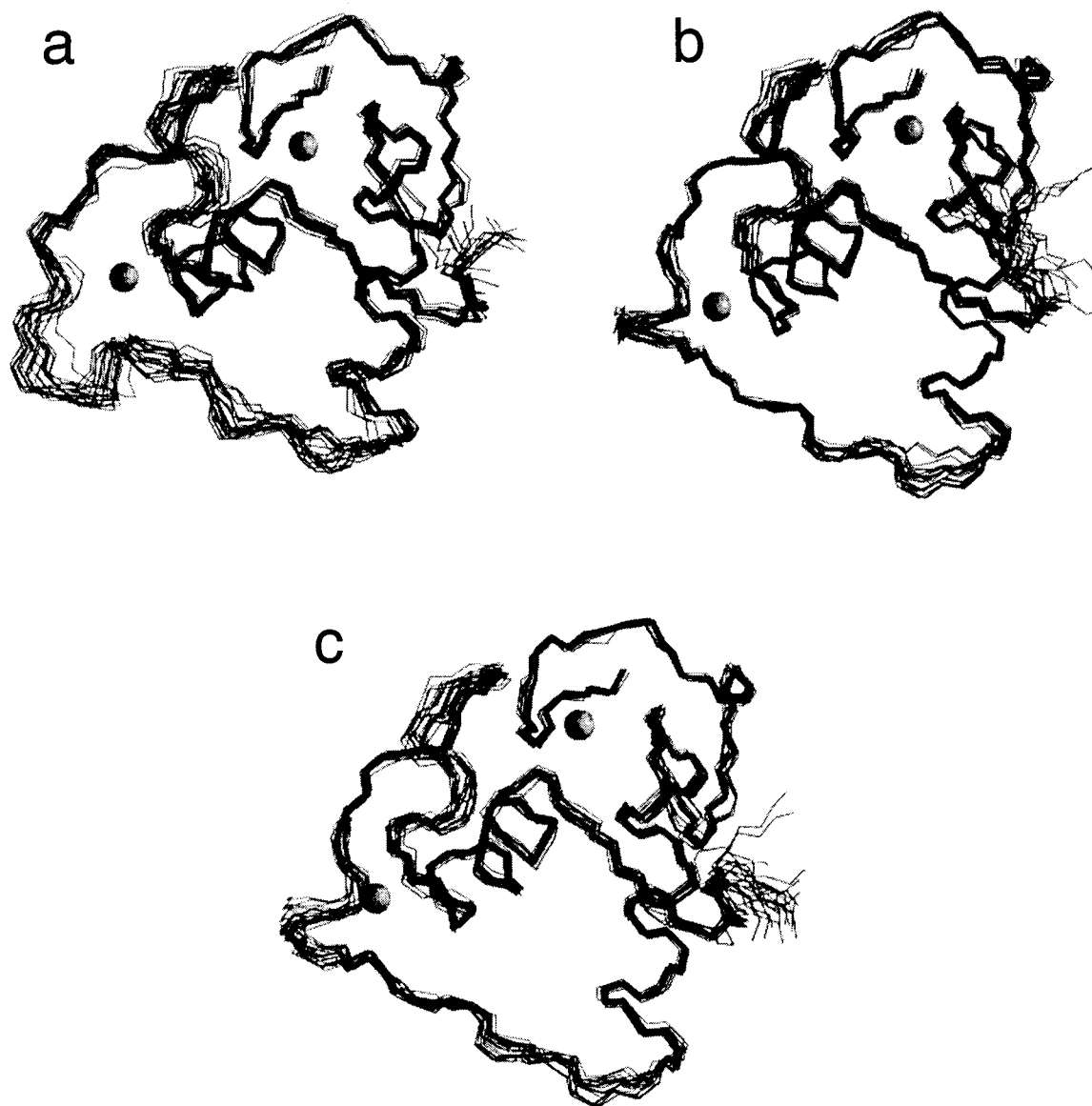


Figure 2. Ensemble structures. The ensemble of structures calculated for (a) the WT, (b) S459A and (c) P493R DBDs.

Phe463 and Pro493. In the free WT structure, the guanido group of Arg496 is in a position to donate a hydrogen bond to the Ser459 γ -oxygen; this hydrogen bond is formed in 50% of the conformers in the NMR ensemble. This hydrogen bond is indicated in Figure 6(a) with a line. Mutation of S459 to A eliminates the γ -oxygen and the potential for hydrogen bonding to Arg496. It is apparent that elimination of this bond is sufficient to induce hydrophobic core reorganization, *via* the close contacts between the arginine side-chain and Pro493 and Phe463; as a part of this process, the χ_1 torsion angle of Arg496 changes from the *g*(+) to the *trans* conformation and the β -carbon at the base of the Arg496 side-chain is displaced towards Pro493 and the D-loop.

We suggest that reorientation of Pro493 is the central event in hydrophobic core reorganization, and that the rigidity imposed on the protein chain by the pyrrolidine ring creates a free energy barrier between two possible states of the hydrophobic core. The orientation of Pro493 seen in the WT free structure may be sterically incompatible with a parallel arrangement of Phe463 and Tyr474. Either by steric clash or by loss of favorable contacts, release of Arg496 from its bond with Ser459 leads to a displacement and flip of Pro493, and, consequently, parallel packing of Phe463 and Tyr474.

This scheme also suggests how the P493R mutation and the act of DNA binding might each induce hydrophobic core reorganization. For P493R, replacement of the rigid pyrrolidine by the

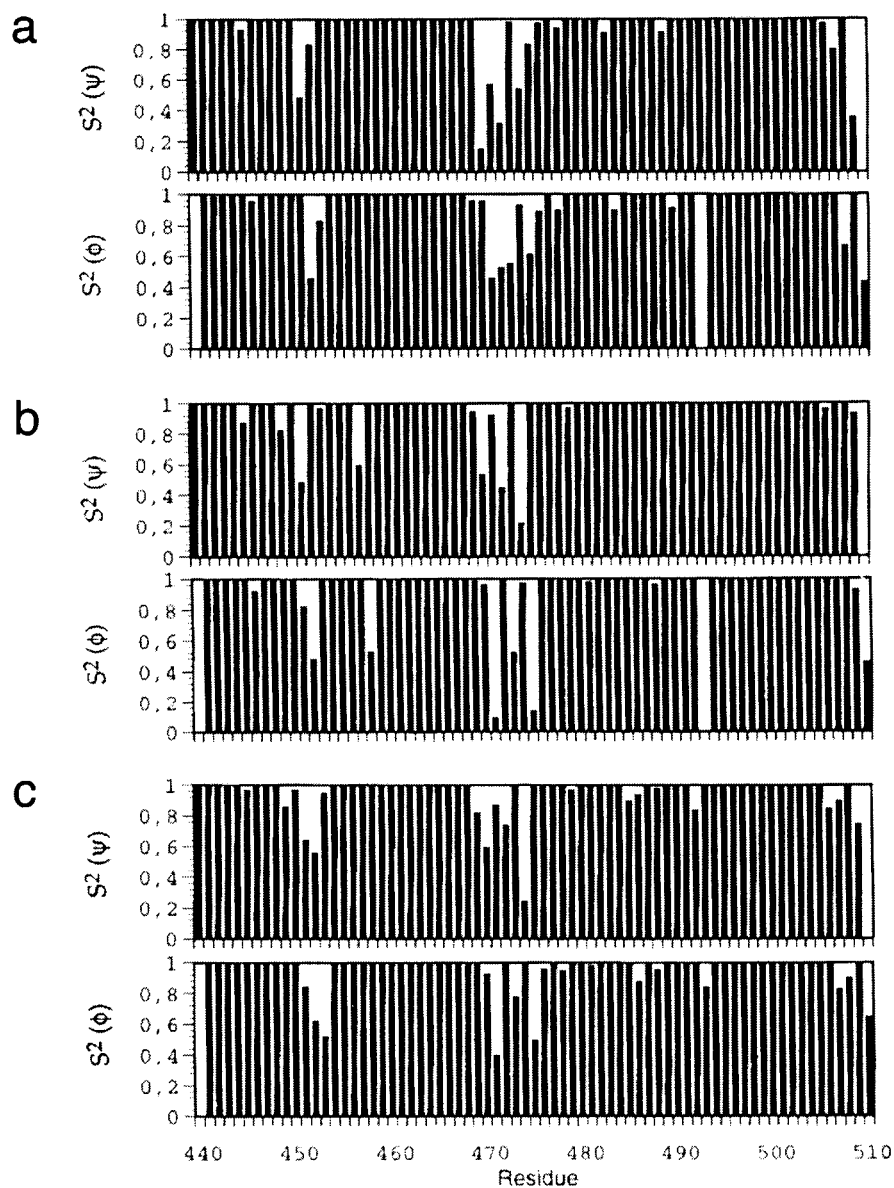


Figure 3. Backbone ψ and ϕ angular order parameters (S^2). (a) WT, (b) S459A and (c) P493R, calculated from the ensemble structures. For a definition of the angular order parameters see Hyberts *et al.* (1992).

flexible arginine side-chain eliminates the barrier to the parallel arrangement of the Phe463 and Tyr474 rings. In the P493R ensemble structures, the aliphatic side-chain of Arg489 descends towards the hydrophobic core to provide additional contacts to the displaced side-chain of Arg496.

In contrast, when the DBD binds to a GRE, phosphate contacts with the DNA backbone may direct hydrophobic core reorganization. In the GRE-bound structure, both Arg489 and Arg496 make phosphate contacts, but Arg489, rather than Arg496, makes a hydrogen bond to the γ -oxygen of Ser459. Either the lost hydrogen bond or the gained phosphate contact might position the Arg496 side-chain to trigger reorientation of Pro493 and reorganization of the aromatic residues

(Figure 6(b)). Tyr474 itself also makes a DNA phosphate contact, which might also favor the parallel positioning of Tyr474 and Phe463.

A conformational relay

The reorganization of the DBD hydrophobic core provides a mechanism by which the mutations or GRE contact induce D-loop reorientation. As shown in Figure 7, the D-loop is fixed at each end by the zinc-coordinating residues Cys476 and Cys482. Immediately, the N terminal of Cys476, aromatic core residue Tyr474 contacts Pro493 in the WT free structure. Upon reorganization of the hydrophobic core, the new packing arrangement of the Pro493 and Tyr474 side-chains requires displa-

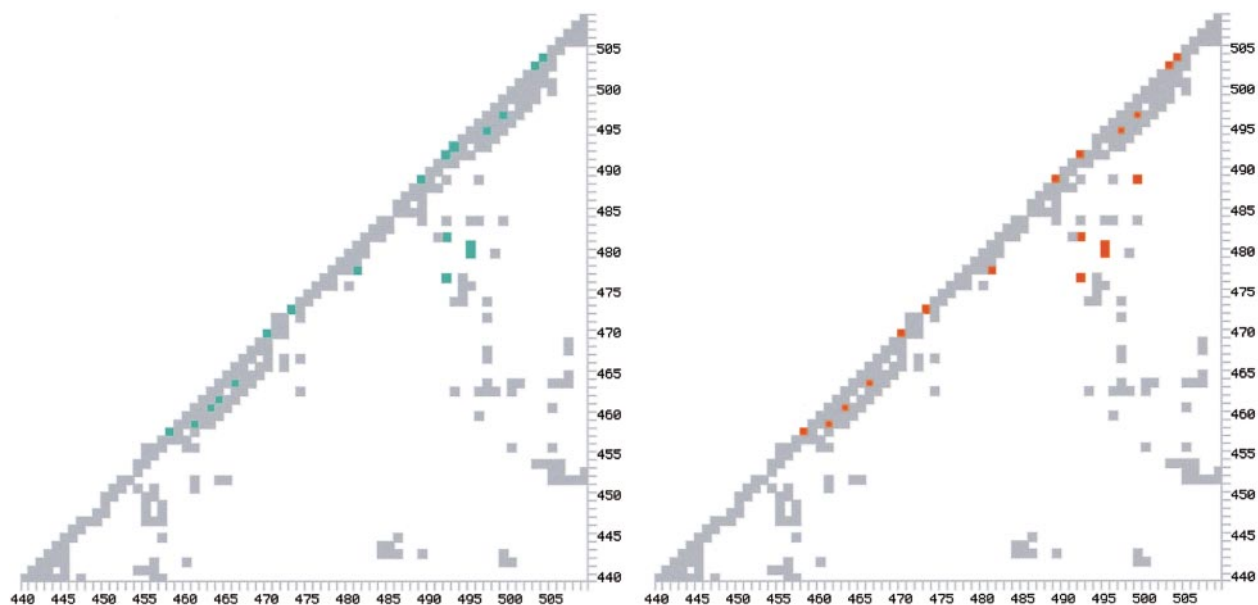


Figure 4. 4 Intra- and inter-residue NOE values. NOE values observed in the spectrum of the free WT DBD are shown in grey. Additional NOE values observed only in the spectrum of the mutant DBDs are shown in green (S459A) and red (P493R).

cement of the backbone in the vicinity of Tyr474 and Leu475. The α -carbon of Cys476 appears to act as a fixed pivot around which the backbone rotates; this rotation is reflected in a transition of the χ_1 torsion angle of Cys476 from the *g*(+) to the *trans* conformation. Rotation of the backbone chain path as it enters Cys476 requires a complementary

rotation as it exits to the D-loop. We suggest that the 474-475 segment acts as a lever arm that swings the succeeding residues of the D-loop into a new orientation through a fulcrum at Cys476.

These inferred reorganizations and displacements appear to comprise a conformational relay, amplifying small changes around the DNA contact

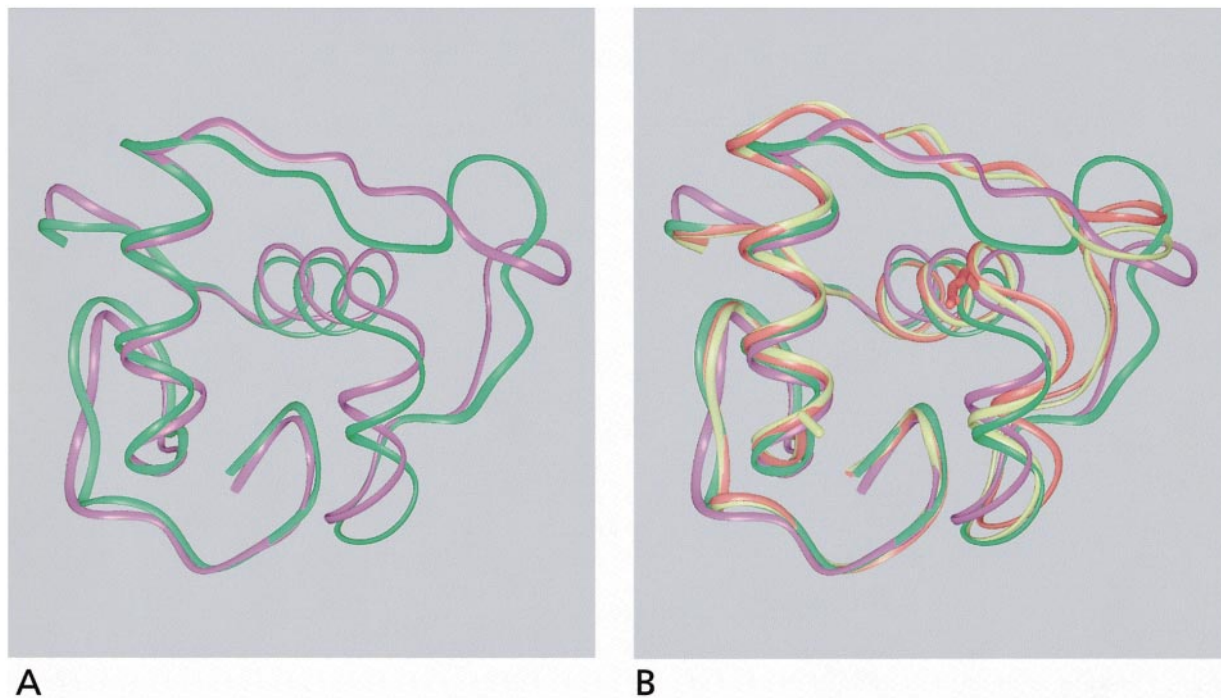


Figure 5. Backbone structures. (a) A representative free WT structure (green) superimposed on the structure of the specifically bound WT DBD from the DBD-GRE complex (purple). (b) Representative structures of the free WT (green), S459A (yellow) and P493R (red) DBDs superimposed on the GRE-bound DBD (purple).

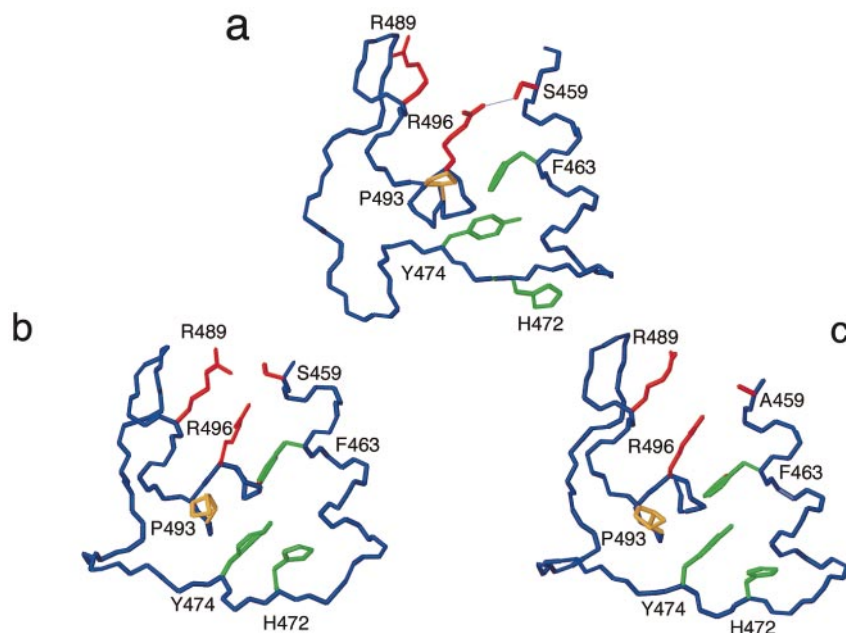


Figure 6. Rearrangement of the DBD hydrophobic core. The structures of the (a) free WT GR DBD, (b) bound WT GR DBD, (c) free S459A GR DBD, are shown from residue Gly458-Arg498. Indicated with a line is the hydrogen bond between the guanido group of Arg496 and the Ser459 γ -oxygen.

surface into a large reorientation of the D-loop. Comparison of the free WT and S459A structures illustrates this principle: between these molecules, the protein conformation at position 459, within the DNA recognition helix, is nearly indistinguishable. Arg496, which has lost its potential to hydrogen bond with Ser459, is shifted towards the D-loop only about 1.5 Å at its β -carbon, the site of maximum displacement. In turn, however, the tip of the Pro493 ring moves about 3.7 Å towards the D-loop, due to the conformational constraints imposed by the proline ring. By contact with Tyr474 and Leu475, this movement is levered through Cys476 to ultimately displace the end of the D-loop over 7 Å in space. Therefore, a minor perturbation in the DNA recognition helix evokes the formation of a protein-protein contact surface over 20 Å away.

Conclusions

DNA as an allosteric effector

The three-dimensional structures of the S459A and P493R mutant DBDs clearly establish that these substitutions give rise to conformational changes that mimic those observed when the DBD binds to a GRE. *In vivo*, the mutant receptors likely dimerize when provided with non-specific DNA as a scaffold for protein-protein assembly (Lefstin *et al.*, 1994). However, the experimental observation of the dimer interface, in the apparent absence of protein dimerization itself, supports strongly the notion that specific DNA contact alone is sufficient to induce conformational change in the DBD. In a similar way, Holmbeck *et al.* (1998) showed that a DNA half-site suffices to induce the dimerization interface in the retinoid X receptor

(RXR). We conclude that DNA indeed acts allosterically to induce conformational changes remote from the contact surface in intracellular receptors.

The ability of the S459A and P493R mutations to mimic the allosteric effect of DNA is reminiscent of certain mutations in prokaryotic transcriptional regulators such as CRP (for a review, see Kolb *et al.*, 1993) and MerR (Parkhill *et al.*, 1993), which mimic the allosteric effects of small molecule ligands, thereby inducing a DNA contact surface. In contrast, the S459A and P493R mutations mimic the allosteric effect of DNA, thereby inducing a protein-protein contact surface. Nonetheless, thermodynamic relationships demand that similar principles govern both situations. Experimental evidence demonstrates that the affinities of CRP and MerR for their small molecule ligands are affected by DNA interaction.

Mechanisms of intramolecular regulation

Although our results establish that the S459A and P493R mutant DBDs constitutively assume the GRE-bound conformation, they leave unanswered the question of how this conformation in turn gives rise to a transcriptional activation surface. Three non-exclusive mechanisms may be considered. First, the DBD itself might form an activation surface capable of contacting a target factor once the GRE-bound conformation is attained. Second, dimerization of GR might create an activation surface in the amino terminus from two previously inactive half-surfaces. Third, long-range intramolecular contacts might transmit conformational changes from the DBD to the amino terminus. The second possibility is consistent with the result that mutation of DBD dimerization contacts eliminated some, but not all, of the mutant pheno-

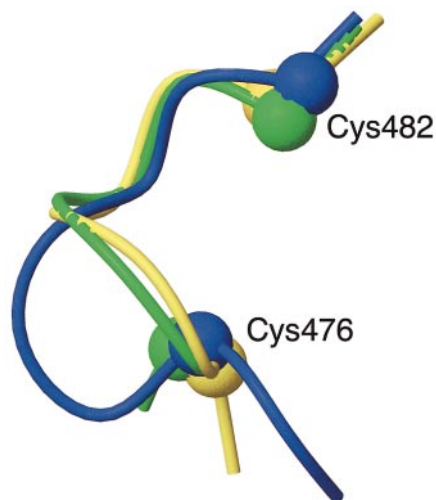


Figure 7. Reorientation of the D-loop. The structures of the free WT DBD (blue), bound GR DBD (green) and the free S459A DBD (yellow) are superimposed. Protein backbones from residue 475 to 483 rendered as a solid ribbon, with α -carbon atoms of Cys476 and Cys482 are shown as solid spheres.

types (Lefstin *et al.*, 1994). However, the third mechanism has been invoked in studies of other transcriptional regulators (Li & Green, 1996; Jonsen *et al.*, 1996) and is consistent with the observation that the GR DBD associates *in vitro* with a conserved region (amino acid residues 96 to 157) of the amino terminus (Lefstin, 1997).

Allosteric regulation by DNA as a general principle

Allosteric regulation of transcriptional regulators by DNA is a common theme among the intracellular receptors. For example, the arrangement of response element half-sites can determine the hormonal response of the RAR-RXR complex (Kurokawa *et al.*, 1995; La Vista-Picard *et al.*, 1996; Mouchon *et al.*, 1999). It is striking in this context that even subtle changes, such as mutation of individual base-pairs within the response element, can elicit distinct conformations of bound intracellular receptors (Staal *et al.*, 1996; Wood *et al.*, 1998) and even convert an activation response into repression. Conversely, mutation of a single amino acid in the DBD recognition helix can switch intracellular receptors from repressors to activators at a range of response elements (Starr *et al.*, 1996). These results imply that intracellular receptors are poised to respond to signals encoded in response elements.

Although the intracellular hormone receptors remain the most compelling example, evidence from other systems indicates that regulatory control by DNA-induced conformational changes may be a general principle in eukaryotic transcriptional regulators (Huang *et al.*, 1998; King *et al.*, 1999; for

a review, see Lefstin & Yamamoto, 1998). The means by which DNA sequence can allosterically modulate the function of transcriptional regulators remain largely unknown. However, the conformational rearrangements we observe in the S459A and P493R hydrophobic cores provide two overlapping mechanisms by which the precise geometry of the protein-DNA interface may influence the conformation of a protein at remote positions: perturbation of the recognition helix, and orientation of amino acid residues that make phosphate contacts. The signal amplification effects we observe in the GR DBD may render the entire protein highly sensitive to variations in response element sequence. We suggest that, in addition to local folding upon DNA binding (Spolar & Record, 1994), rearrangement of hydrophobic cores upon DNA binding (Ogata *et al.*, 1996) may provide a mechanism by which information embedded in a DNA site is transmitted to the protein-protein contact surfaces of transcriptional regulators.

Materials and Methods

Purification and sample preparation of ^{15}N -labeled GR DBD

The DBD fragments (kparpC440-K513) were overexpressed using the plasmid pT7-7 in *Escherichia coli* strain BL21[DE3]/pLysS. Uniformly ^{15}N -labeled proteins were obtained by growing the recombinant strain in a minimal medium with $[^{15}\text{N}]\text{NH}_4\text{Cl}$ (Cambridge Isotope) as the only nitrogen source and with 100 mg/ml ampicillin and 40 mg/ml chloramphenicol at 37°C. Protein expression was induced by addition of 0.5 mM IPTG at an A_{600} of 0.7-1.0. Three hours after induction, the cells were collected by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 1 mM EDTA, 500 mM NaCl, 40 mM MgCl_2 , 5 mM DTT and 0.5 mM PMSF (pH 8) before purification. The lysate was then precipitated with ammonium sulfate at 70% saturation. The precipitate was collected and resuspended in 10 mM Tris-HCl (pH 8.0), 5% glycerol, 0.1 mM EDTA, 50 mM NaCl, 1 mM DTT, 50 mM ZnCl_2 , 0.5 mM PMSF and dialyzed against this buffer. The lysate was then loaded on an Accell column and eluted with a linear NaCl gradient (50 mM-1 M) of 20 mM phosphate buffer at (pH 7.6) and 1 mM DTT. The pooled peak fractions were further purified on a HiLoad Superdex 75 prep grade FPLC column (Pharmacia) with 20 mM phosphate buffer (pH 7.6), 1 mM DTT and 150 mM NaCl. The pooled fractions were concentrated using a 3 K Macrosep (Filtron). Protein concentrations were determined spectrophotometrically using $\epsilon_{280\text{ nm}} = 4200\text{ M}^{-1}\text{ cm}^{-1}$ calculated for tyrosine absorption (Cantor & Schimmel, 1980).

NMR samples in a 95%/5% $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ mixture were prepared by dialysis with a 3 K Macrosep (Filtron) against 150 mM NaCl, 1 mM DTT (pH 7.6 and 6.8). Trace amounts of NaN_3 were added to all samples. Each sample contained approximately 3 mM of protein and was bubbled with He gas to remove dissolved oxygen. All three protein samples were prepared with the same buffer batch to eliminate differences in sample conditions.

NMR spectroscopy

NMR spectra were recorded on a Varian Unity plus 750 MHz spectrometer and a Bruker AMXT 600 MHz spectrometer at 298 K. Proton chemical shifts are referred to the water signal at 4.75 ppm. The following 2D homonuclear NMR experiments were performed: nuclear Overhauser enhancement spectroscopy (NOESY) (Jeener *et al.*, 1982), with mixing times of 50, 75 and 150 ms; total correlated spectroscopy (TOCSY) (Griesinger *et al.*, 1988) with mixing times of 20, 35 and 70 ms. NOESY-heteronuclear single quantum coherence 3D (NOESY-HSQC) (Marion *et al.*, 1989) with a mixing time of 100 ms and (3D HNHA) (Vuister & Bax *et al.*, 1993) were recorded to solve the overlap problem and to determine $^3J_{\text{HNH}\alpha}$ coupling constants, respectively. The saturation transfer experiment was performed by recording two [^1H - ^{15}N] HSQC spectra, one with and one without water flip-back. All the NMR data were processed using the TRITON NMR program package on Silicon Graphics INDY and INDIGO2 workstations.

Structural constraints

^1H and ^{15}N resonances were assigned using the standard methods of Wüthrich (1986) and Marion *et al.* (1989), and compared with the initial assignment of the 440-525 DBD fragment (Härd *et al.*, 1990a,b). Based on cross-peak volumes from the NOESY spectra recorded with a mixing time of 75 ms, the NOEs were quantified as distance constraints. Calibration was based on observed intraresidue NOEs between CH₂,6 and CH₃,5 protons of the tyrosine residues. These distance constraints were divided into three classes: strong (<2.7 Å), medium (<3.3 Å) and weak (<5 Å) with a lower distance limit set to the sum of the van-der-Waals radii (1.8 Å). NOE values found only in the NOESY spectra with a mixing time of 150 ms were set to 5 Å as distance constraint. Pseudo-atoms corrections were added according to Wüthrich (1986) except for methyl groups, for which a value of 0.3 Å was used (Koning *et al.*, 1990). Hydrogen bonding constraints were not included in the structure calculations. Twelve sulfur-sulfur distance constraints between the zinc-coordinated cysteine residues were implemented assuming a Zn-S distance of 2.35 Å and tetrahedral coordination, with a lower and upper bound of 3.80 Å and 3.85 Å, respectively. Backbone ϕ angles were derived from a 3D HNHA experiment (Vuister & Bax, 1993). The ratio between the intensities of the cross-peak $\text{H}_\text{N}\text{-H}_\alpha$ and the diagonal peak was determined and from this backbone ϕ angles were derived. Side-chain $\text{H}^\alpha\text{-H}^\beta$ coupling constants were estimated from TOCSY spectra and compared with the corresponding intensities of the $\text{H}^\alpha\text{-H}^\beta$ and $\text{H}_\text{N}\text{-H}^\beta$ NOE cross-peaks in the 50 ms NOESY spectrum to derive χ_1 angles. Methyl groups of valine residues were assigned stereospecifically using the method described by Zuiderweg *et al.* (1985).

Structure calculations

Structure calculations and analysis were carried out on a Silicon Graphics workstation using InsightII 95.0.1 (MSI, San Diego, CA, USA). The structures of the three proteins were all generated with distance geometry (Havel, 1983) using DGII (Havel, 1991). For each protein a set of 25 structures was generated. The structures were refined using the program Discover (BIOSYM Technologies, San Diego, CA, USA) with the following protocol: steepest descent energy minimization (100 iterations); conjugated

gradients minimization (100 iterations), followed by restrained molecular dynamics simulations *in vacuo* (10,000 iterations of 1 femtosecond) and subsequently, steepest descent and conjugated gradients minimization (100 and 500 iterations, respectively). All distance geometry calculations and restrained MD simulations were performed on a Silicon Graphics Challenge computer.

Acknowledgments

This work was supported by the Chemistry division of the Netherlands Organization for Scientific Research (NOW-CW), and by grants from the US National Science Foundation and National Institutes of Health. The 750 MHz NMR spectra were recorded at the SON NMR Large-Scale Facility in Utrecht, which is supported by the Large-Scale Facility program of the European Union. The authors thank A. George for help with the initial overexpression and protein purification.

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Edited by P. E. Wright

(Received 8 June 2000; accepted 29 June 2000)



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