NMR investigation of the interaction of the inhibitor protein Im9 with its partner DNase

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Abstract

The bacterial toxin colicin E9 is secreted by producing *Escherichia coli* cells with its 9.5 kDa inhibitor protein Im9 bound tightly to its 14.5 kDa C-terminal DNase domain. Double- and triple-resonance NMR spectra of the 24 kDa complex of uniformly ¹³C and ¹⁵N labeled Im9 bound to the unlabeled DNase domain have provided sufficient constraints for the solution structure of the bound Im9 to be determined. For the final ensemble of 20 structures, pairwise RMSDs for residues 3–84 were 0.76 ± 0.14 Å for the backbone atoms and 1.36 ± 0.15 Å for the heavy atoms. Representative solution structures of the free and bound Im9 are highly similar, with backbone and heavy atom RMSDs of 1.63 and 2.44 Å, respectively, for residues 4–83, suggesting that binding does not cause a major conformational change in Im9. The NMR studies have also allowed the DNase contact surface on Im9 to be investigated through changes in backbone chemical shifts and NOEs between the two proteins determined from comparisons of ¹H–¹H–¹³C NOESY-HSQC spectra with and without ¹³C decoupling. The NMR-defined interface agrees well with that determined in a recent X-ray structure analysis with the major difference being that a surface loop of Im9, which is at the interface, has a different conformation in the solution and crystal structures. Tyr54, a key residue on the interface, is shown to exhibit NMR characteristics indicative of slow rotational flipping. A mechanistic description of the influence binding of Im9 has on the dynamic behavior of E9 DNase, which is known to exist in two slowly interchanging conformers in solution, is proposed.

Keywords: colicin; DNase; immunity protein; NMR; protein–protein interactions

Colicins form an integral part of the stress response system of *Escherichia coli* by killing sensitive cells in their environment (James et al., 1992, 1996). To protect the producing cell against the action of the toxin, an immunity protein is co-produced with the colicin. This binds to the colicin and inhibits its killing action. Our work concerns the family of E-type colicins with DNase activity. These comprise the ~61 kDa colicins E2, E7, E8, and E9, which have ~14.5 kDa DNase domains at their C-terminal ends, and their respective ~9.5 kDa immunity proteins Im2, Im7, Im8, and Im9 (Jakes & Zinder, 1974; Sidikaro & Nomura, 1974; Wallis et al., 1992a, 1992b; James et al., 1996). The cognate complexes

formed by these proteins have extremely high binding affinities: for example, the complexes formed between ColE9 or the E9 DNase domain with Im9 have a K_d of $\sim 10^{-16}$ M (Wallis et al., 1995b). Furthermore, though noncognate complexes between the E9 DNase and Im2, Im7, or Im8 are formed, they have significantly weaker binding affinities as shown by their dissociation constants of $\sim 10^{-4}$ M for Im7–E9 DNase, $\sim 10^{-6}$ M for Im8–E9 DNase and $\sim 10^{-8}$ M for Im2–E9 DNase (Wallis et al., 1995a). This range of K_d values spanning 12 orders of magnitude makes this a powerful system for studying factors governing affinity and specificity of interprotein interactions.

To address the issues of affinity and specificity in this system, structural information for both the free and the complexed proteins is needed. So far, we have obtained structures for the free immunity proteins Im9 and Im7 by NMR spectroscopy (Osborne et al., 1994, 1996) and X-ray crystallography (Dennis et al., 1998), respectively. The latter has also been determined independently by Chak et al. (1996). Free E9 DNase has been investigated by NMR spectroscopy (Whittaker et al., 1998, 2000), but a full structure determination has not been reported. Site-directed mutagenesis of this DNase has identified active site residues for DNA hydrolysis

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Abbreviations: CSI, chemical shift index; ColE9, colicin E9; E9 DNase, expressed C-terminal 134 amino acids of colicin E9 encompassing the DNase activity; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; HSQC, heteronuclear single quantum coherence; Im9, immunity protein of colicin E9; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ppm, parts per million; RMSD, root-mean-square deviation; TOCSY, total correlation spectroscopy.

(Garinot-Schneider et al., 1996) and recent X-ray structure determinations of the Im9–E9 DNase and Im7–E7 DNase complexes (Kleanthous et al., 1999; Ko et al., 1999) have addressed the issue of how the immunity proteins act as inhibitors. These bind to their DNase at a region remote from the active site and inhibit the DNase by preventing DNA binding through a combination of steric and electrostatic effects. The X-ray structures also reveal that the DNase domains contain a transition metal ion binding site >15 Å from the bound Im9. Since a transition metal ion is not essential for immunity protein binding or DNA hydrolysis (Pommer et al., 1999), a biological function for this site is not known.

The colicin DNase domains share $\sim 65\%$ sequence identity while their immunity proteins are $\sim 50\%$ identical to each other (Lau et al., 1992), leading to strong structural similarities. Im7 and Im9 are four-helix bundles consisting of three long helices (I, II, and IV) and a shorter helix (III). NMR and mutagenesis data (Li et al., 1997, 1998; Osborne et al., 1997; Wallis et al., 1998) show that the main contribution to the binding interaction results from helix II, which is highly variable, and helix III, which is conserved within the family of immunity proteins. The combination of a variable specificity determining region and a common binding region is the basis of the dual-recognition mechanism for determining specificity among the colicin DNase-Im complexes (Li et al., 1998; Wallis et al., 1998).

There are several complications with comparing the crystal structure of the Im9–E9 DNase with the solution NMR data. The first is that the X-ray structure only detects a single conformation for the bound DNase (Kleanthous et al., 1999; Kühmann et al., 2000) while the NMR studies indicate the presence of two slowly interchanging conformers for both the free and the bound E9 DNase (Whittaker et al., 1998, 2000). Second, comparison of the NMR structure of the unbound Im9 with the X-ray structure of the bound Im9 shows structural differences between the two, most notably in the loop connecting helices III and IV. These observations have prompted us to determine the structure of Im9 bound to the metal-free E9 DNase by NMR spectroscopy. In addition, although the X-ray structures of the cognate Im9–E9 DNase and Im7–E7 DNase complexes (Kleanthous et al., 1999; Ko et al., 1999) are consistent with the dual-recognition mechanism for specificity determination, the structures of noncognate complexes such as Im7–E9 DNase and Im2–E9 DNase are needed to provide a full structural description of this. Since crystallization trials of such mixed complexes have failed to provide suitable quality crystals (A. Hemmings, unpubl. results), NMR studies of the type reported here are needed to investigate structural differences between cognate and noncognate complexes, and this characterization of the Im9–E9 DNase complex provides a benchmark for this.

Results and discussion

Backbone and side-chain assignments of bound Im9

Despite the E9 DNase adopting two slowly exchanging conformations in solution, all spectra of the complex containing doubly labeled Im9 showed the presence of only one Im9 species. This indicates that the slow chemical exchange of the nuclease did not induce a similar phenomenon in the immunity protein.

HNCA, HNCO (Ikura et al., 1990a; Kay et al., 1990), and CBCA(CO)NH (Grzesiek & Bax, 1992) spectra provided the sequential backbone assignment for all residues apart from the N-terminal methionine. Side-chain assignments were obtained from

an HBHA(CBCACO)NH (Grzesiek & Bax, 1993) spectrum, which gave $\sim 50\%$ of the α and β proton assignments, and HcCH- and hCCH-TOCSY experiments (Bax et al., 1990) with a 22.5 ms mixing time. The majority of the aliphatic side-chain assignments was extracted from the hCCH-TOCSY spectrum since its signal:noise ratio was far superior to that of the HcCH-TOCSY spectrum. The HBHA(CBCACO)NH and HcCH-TOCSY experiments, where proton magnetization evolved during one of the incremented time periods, proved inferior in terms of sensitivity to the hCCH variant with transverse ^{13}C magnetization in both indirect dimensions. Presumably, this was because the size and shape of the complex resulted in unfavorable ^1H transverse relaxation times while carbon T_2 values were not so affected. The assignment of resonances of the aromatic side chains from NOESY spectra alone was difficult, partly because many of the signals were either broadened and/or weak, sometimes to the extent of being unobservable, or overlapping. Since the dedicated aromatic HBCBCGCDHD experiment (Yamazaki et al., 1993) only yielded a good signal:noise ratio for residues already identified, an HcCH-TOCSY spectrum centered in the aromatic carbon region at 114 ppm was measured to obtain ^{13}C chemical shifts.

Resonances of the four ring protons of Tyr54 were observed at 6.80/131.93 ppm ($^1\text{H}/^{13}\text{C}$), 6.61/130.46 ppm for the H_δ , and 6.20/116.20 ppm, 5.32/115.96 ppm for the H_ϵ protons, indicating that the fast flipping between equivalent orientations normally observed for tyrosine rings, and seen for Tyr54 of the unbound Im9 (Osborne et al., 1994), had been prevented by the interaction of Im9 with E9 DNase.

The structure of Im9 bound to E9 DNase

The chemical shifts extracted from the triple resonance data allowed the prediction of secondary structure elements using the Chemical Shift Index (CSI) (Wishart et al., 1992). The consensus CSI of C_α , CO, and H_α chemical shifts yielded helices for residues 12–23, 30–44, and 69–77. The sequential NOEs (Fig. 1) and final DYANA/OPAL calculations, using 2,058 meaningful structural constraints (Table 1), are in agreement with this prediction for helices I, II, and IV, and they also identify the short helix III for residues 49–51 (Fig. 2A).

The agreement within the family of 20 OPAL structures is generally good (Table 2; Fig. 2B), with an RMSD to the mean structure of 0.67 Å for the backbone atoms, and pairwise RMSDs for residues 3–84 of 0.76 ± 0.14 Å for the backbone and 1.36 ± 0.15 Å for all heavy atoms. The sequence variation of backbone and heavy atom RMSDs (Fig. 3A) reflects the number of structurally significant NOEs available for a residue (Fig. 3B), as illustrated by the relatively large RMSD observed for helix III (Fig. 3A). In this case, NOESY cross peaks are often very weak due to the interaction with E9 DNase. The regions of the structure most poorly defined are, apart from the termini, the interhelical loops and the end of helix IV (Fig. 3A). Loop 1 forms part of the interface where many resonances are weak, therefore making the detection of NOEs difficult. The lack of definition at the end of helix IV results from the fact that the peaks of Arg75 are weak to unobservable in all spectra. Due to the lack of sequential NOEs from Arg75, the final turn of helix IV opens prematurely, limiting the length of the helix. Analysis of the Ramachandran plot for the ensemble of 20 structures shows more than 90% of all residues in the most favored and additional allowed regions, with 3.4% being located in the disallowed areas. No residue is found in a disallowed

Fig. 1. Summary of sequential and medium-range NOE connectivities for Im9 bound to E9 DNase. The intensities of sequential NOEs are categorized as strong, medium, and weak, and are represented by the thickness of the lines.

region in all of the 20 structures, and only two residues were present in these regions in more than 10 of the structures, Ser29 and Lys57. These are located in loops 1 and 2, respectively. Furthermore, Ser29 also forms part of the interface with E9 DNase. It

Table 1. Summary of NMR parameters for structure calculations

Restraint type	Number of restraints
Intraresidue NOEs	330
Interresidue sequential NOEs ($ i - j = 1$)	542
Interresidue short-range NOEs ($1 < i - j < 5$)	556
Interresidue long-range NOEs ($ i - j = 5$)	615
Total number of NOEs	2,043
Hydrogen bond restraints (one per H-bond)	15
Total number of restraints	2,058

has been observed before that residues in loop regions can adopt nonstandard conformations (Lubienski et al., 1994) and the fact that an interface residue has unfavorable backbone dihedral angles could indicate that this particular geometry might be required to enable a necessary interaction on the interface. However, Ser29 does not have unusual backbone angles in the X-ray structure. This is reminiscent of structural studies of barstar; several residues have positive ϕ angles in the solution structure, but only one maintains this angle in the X-ray structure (Buckle et al., 1994; Lubienski et al., 1994).

Comparison of the NMR structures for free and bound Im9

The representative solution structures of free and bound Im9 are highly similar with backbone and heavy atom RMSDs of 1.63 and 2.44 Å for residues 4–83 indicating no major conformational change upon binding. The helices have backbone/heavy atom RMSDs of 0.45/1.01 Å for helix I, 0.68/1.69 Å for II, 0.20/1.13 Å for III, and 0.86/1.31 Å for helix IV. The comparatively large RMSD for

Fig. 2. **A:** Ribbon model indicating the secondary structure elements (generated in MOLMOL (Koradi et al., 1996)). The overall length of each helix is slightly shorter than in the free protein, helix I ranging from Glu12–Glu22 compared to Glu12 to Asp24 in free Im9, helix II including residues 32–43 (30–44), helix III comprising Gly49, Ser50, and Asp51 (51–55) while residues 66–77 (64–78) form helix IV. **B:** A worm representation of the overlay of the family of 20 structures of the bound Im9. The width and shading are representative of the RMSD values for each residue relative to the average structure (created in MOLMOL). Black indicates residues with an average RMSD value >0.89 Å, dark grey RMSD values >0.50 Å, and light grey indicates residues that have RMSD values <0.50 Å. The average global backbone RMSD values for residues 3–84 are given in Table 2.

helix IV results from a slight reorientation of helix III and loop 2 upon binding. The biggest differences between the free and bound Im9 structures are in loop 2 and the termini, which are poorly defined within both families of NMR structures because of the low number of NOEs for these regions (Fig. 3B), and loop 1, which is part of the binding interface where some rearrangement could take place to accommodate interaction with its binding partner. In general, the helices are slightly shorter than in the free protein (Fig. 2A), probably because residues 28–31, located just before the start of helix II, and residues on helix III are involved in binding to the DNase. As a result, some of their resonances have fewer intra-molecular NOEs than in the free Im9, leading to a lack of NOE constraints, and thus definition of the helical elements.

Table 2. Summary of structural statistics

Quantity	Value
OPAL energy (kJ mol ⁻¹)	-2,335.55 ± 60.12
Residual distance constraint violations	
Number > 0.4 Å	0
Sum (Å)	31.098 ± 0.41
Maximum (Å)	0.13 ± 0.01
Pairwise RMSDs (Å)	
Backbone atoms of residues 3–84	0.76 ± 0.14
All heavy atoms of residues 3–84	1.36 ± 0.15

Although the overall structure comparison for free and bound Im9 indicates that there is not a major conformational change upon binding, we considered it possible that the interaction of Im9 with E9 DNase could give rise to either slight changes in helix angles and/or the reorientation of some amino acid side chains, and therefore undertook a detailed analysis to investigate this. The inter-helix angles for the free and bound ensembles of 21 and 20

Fig. 3. **A:** Distribution of the atomic RMSDs determined over the 20 solution structures of bound Im9 relative to the average structure for the backbone atoms (solid line) and heavy atoms (dotted line). Helical regions are indicated by filled symbols. **B:** Histogram showing the number of structurally significant NOEs per amino acid residue. White indicates intra-residue, light grey sequential, dark grey medium-range, and black long-range constraints.

Table 3. Helix angles for free and bound Im9

	I-II	I-III	I-IV	II-III	II-IV	III-IV
Free Im9	144.7 ± 1.2°	64.8 ± 2.7°	132.9 ± 3.1°	129.7 ± 1.3°	20.3 ± 1.6°	148.9 ± 1.5°
Bound Im9 (NMR)	142.2 ± 1.9°	64.8 ± 9.8°	137.9 ± 3.3°	119.3 ± 8.0°	21.2 ± 1.9°	139.9 ± 6.8°
Bound Im9 (X-ray)	142.1°	58.0°	128.6°	115.9°	32.0°	142.4°

structures, respectively, were not significantly different (Table 3), though the standard deviations for the bound protein are higher than those in free Im9. For differences in side-chain orientations between free and bound Im9 to be significant, they have to be greater than the spread of conformations within the families of structures, and the side chain has to be on or near the interface. The main regions with the greatest difference between free and bound Im9, apart from the termini, are the loop regions that also have large side-chain RMSDs in the free and the bound protein. This is similar for other proteins in their free and bound states (Lo Conte et al., 1999). Some side chains with sufficient intra- and inter-residue NOEs to be well defined, but still with large RMSDs, do not belong to surface residues for which conformational flexibility is to be expected, but rather to buried residues not located on the interface with the DNase e.g., Ile7, Phe15, and Met43. Their RMSDs may simply indicate flexibility of longer side chains held in hydrophobic pockets. Residues that have significantly different orientations in the free and bound Im9 include: Glu24, Ser28, Glu30, Leu52, Ile53, Pro56, and Lys57. The latter two are part of loop 2, where large differences between side-chain orientations can be expected because of the small number of NMR constraints (Fig. 3B). Glu24, Ser28, and Glu30 are polar surface residues that are highly accessible in the free protein, so large RMSDs are in agreement with their surface exposure. Leu52 and Ile53, however, are hydrophobic residues with well-defined conformations that are buried in the free protein. Thus, for these residues, a change in side-chain position on binding DNase is indicated. We consider Ile53 below when we discuss the nature of the Im9 binding surface and the conformational dynamics of the bound DNase.

Comparison of NMR and X-ray crystallographic structures of bound Im9

Comparison of the structure of Im9 bound to E9 DNase obtained by NMR spectroscopy with that determined by X-ray crystallography yields results similar to those for comparison of the free and bound structures obtained by NMR. The backbone RMSD for residues 3–85 is 1.56 Å, but for the helices alone even better agreement is found: helix I yields an RMSD of 0.43 Å, II 0.55 Å, III 0.33 Å, and helix IV 0.65 Å. The helix angles for the bound protein determined by X-ray crystallography are almost identical to those for the NMR structure. As with the comparison of NMR structures, the main differences lie in the termini and the loops, particularly in loop 2, which is in a different orientation in the crystal structure. Since loops generally do not adopt a fixed geometry, it may not be surprising to find different conformations in this part of the molecule but it is significant as the X-ray structure indicates that there is a hydrogen bond between the side chains of Asp62 of Im9 and Asn72 of the DNase.

The unusual high field shift of one of the ϵ protons of Tyr54 in the complex suggests a ring-current shift from another aromatic

system, and the strong NOEs between the H_ϵ resonance of Tyr54 and E9 DNase are consistent with this. Inspection of the X-ray structure confirmed the close proximity of an aromatic group in the DNase, Phe86 (Fig. 4). The interaction of the two rings is neither the common T-type arrangement with one ring perpendicular to the other (Linse, 1992), nor the formation of a hydrogen bond between the OH group of one ring and the π system of the second. Rather unusually, the two aromatic rings are almost parallel to each other, with an angle of about 20° between them, and the centers 4.4 Å apart and slightly shifted relative to each other (Kleanthous et al., 1999). Chemical shift calculations using the Total program (Williamson & Asakura, 1993; Asakura et al., 1995) gave quantitative agreement between the X-ray and NMR structures as far as the environment of Tyr54 is concerned. The $H_{\epsilon x}$ and $H_{\epsilon y}$ resonances of Tyr54 had chemical shifts of 5.32 and 6.80 ppm, respectively, while the $H_{\epsilon y}$ and $H_{\delta y}$ resonances were at 6.20 and 6.61 ppm. Calculations based on the X-ray structure gave the chemical shifts of the four ring protons as 5.60, 6.70, 6.40, and 6.75 ppm, respectively.

Tyrosine and phenylalanine residues generally flip rapidly through 180° about their C_β - C_γ bonds. Whether the aromatic side-chain resonances are in fast, slow, or intermediate chemical exchange depends on the rate at which flipping occurs and the NMR frequency difference across the ring. For Tyr54 of the bound Im9, the chemical shift difference of 0.4 ppm between the two H_ϵ resonances corresponds to an upper rate of flipping of 1,200 s⁻¹ at an operating frequency of 500 MHz. The actual rate is likely to be considerably lower than this, however, since Tyr54 in the complex is completely buried within the interface and the ring flip needs to be accompanied by motions of residues surrounding the ring. How-

Fig. 4. Ribbon diagram (generated in MOLMOL (Koradi et al., 1996)) of the interface between Im9 (yellow) and E9 DNase (red) taken from the X-ray structure (Kleanthous et al., 1999) indicating the interaction between Tyr54 of Im9 and Phe86 of E9 DNase.

Fig. 5. Weighted average ($\Sigma|\Delta\delta|/\Delta_{max}$) of HN, N, and C_α chemical shift differences between free and bound Im9 as a function of residue number.

ever, for Tyr55, which also forms part of the interface, flipping on the fast exchange timescale does occur. An important factor here is that Tyr55 is not close enough to another aromatic ring to experience a large chemical shift perturbation, but it may also be important that the ring of Tyr55 has one face exposed at the surface of the complex and interacts with the DNase through a hydrogen bond to its OH rather than through interactions with its aromatic ring (Kleanthous et al., 1999; Kühlmann et al., 2000). Such hydrogen bonds do not hinder motion about the C_β - C_γ bond as they lie along the C_2 symmetry axis.

The interaction surface on Im9 for the E9 DNase

The NMR studies have allowed the DNase contact surface on Im9 to be determined in solution (Fig. 7) through changes in chemical shifts (Fig. 5) and direct NOEs between the two proteins (Fig. 6). Changes in ^1H and/or ^{15}N chemical shifts for a labeled protein in the presence and absence of its binding partner is a common method of identifying potential sites of interaction (Osborne et al., 1997; Rajagopal et al., 1997). Applying this to Im9 binding to E9 DNase using a weighted average ($\Sigma|\Delta\delta|/\Delta_{max}$) of HN, N, and C_α chemical shift differences shows the interaction region involves helices II and III as well as loops 1 and 2 (Fig. 5). The region mapped by chemical shifts agrees well with the X-ray structure (Kleanthous et al., 1999; Kühlmann et al., 2000) and is supported by alanine scanning mutagenesis (Wallis et al., 1998), which identifies the residues with the largest contributions to binding as Leu33 and Val34 from helix II, and Asp51, Tyr54, and Tyr 55 from helix III.

Observation of intermolecular NOEs should be a better approach for mapping intermolecular interactions than chemical shift perturbations and a technique that has been used for interprotein and peptide-protein complexes involves the acquisition of an isotope-labeled, isotope-filtered NOESY experiment (Ikura et al., 1990b; Zuiderweg et al., 1990). This makes use of ^{13}C labeling in one protein while leaving its binding partner unlabeled. However, application of this method did not yield any cross peaks for the Im9-DNase complex and to identify intermolecular NOEs we mea-

sured ^{13}C -edited three-dimensional (3D) NOESY spectra with and without carbon decoupling during acquisition. This resulted in all intramolecular peaks appearing as doublets due to $^1J_{\text{CH}}$ splitting in the spectrum without decoupling while the cross peaks originating

Fig. 6. Strips for the H_a of Asn24, taken from the ^1H - ^1H - ^{13}C -NOESY-HSQC experiment with and without decoupling during acquisition. Intermolecular NOEs are singlets in both strips but intramolecular NOEs for Im9 are doublets in the coupled spectrum.

Fig. 7. (A) Ribbon and (B) accessible surface representations of bound Im9 (generated in SPOCK (Christopher & Baldwin, 1998)): pale yellow indicates residues with weighted average chemical shift differences >0.39 ppm upon binding; orange shows residues whose intramolecular NOEs reduce in intensity upon binding; red indicates residues whose resonances are shifted significantly on binding, exhibit strong NOEs and show NOE contacts to E9 DNase.

from interactions with unlabeled E9 DNase showed up as singlets (Fig. 6). Comparison of coupled and decoupled ^{13}C NOESY spectra revealed 129 intermolecular cross peaks. However, the resulting information could not be fully exploited due to the lack of complete side-chain assignments and availability of an NMR structure of E9 DNase in the complex, which are a consequence of the spectral complexity resulting from the two slowly interchanging DNase conformers (Whittaker et al., 2000).

The Im9 residues showing NOEs to the E9 DNase are Cys23, Asn24, Ala25, Asp26, Thr27, Ser29, Glu31, Leu33, Val34, Val37, Ser48, Gly49, Ile53, and Tyr54, all of which are within 5.0 Å of the DNase in the X-ray structure. The crystal structure shows an additional 12 Im9 residues within 5.0 Å of the DNase, none of which show intermolecular NOEs: Ile22, Ser28, Glu30, Thr38, Glu41, Pro47, Ser50, Asp51, Tyr55, Pro56, Asp62, and Ile67. Of these, resonances of Ile22, Ser28, Glu30, Thr38, Ser50, Asp51, Tyr55, and Pro56 are significantly shifted on DNase binding (Fig. 5), indicating that they are indeed at the interface. The lack of intermolecular NOEs for these groups suggests that there is some feature of the interprotein complex that reduces the cross-peak intensity. As all of these residues are close to interface water molecules (Kühlmann et al., 2000), it may be the presence of tightly bound water that is responsible for the lack of NOE intensity through providing an alternative cross-relaxation pathway for the Im9 protons.

Glu41, Pro47, Asp62, and Ile67 do not show intermolecular NOEs but they do have sizable intramolecular NOEs, unlike those Im9 groups close to interfacial water. Since these groups do not have significant complexation induced changes in chemical shifts (Fig. 5), it is likely that they are not firmly located at the boundary of the interface and bulk solvent. In this context, it may be important that Glu41 and Ile67 are at the ends of helices and Pro47 and Asp62 in interhelical loops. The difference between the NMR and X-ray data for Asp62 is surprising considering that there is a hydrogen bond between the side chains of Asp62 of Im9 and Asn72 of the DNase in the Im9–E9 DNase X-ray structure (Kleanthous et al., 1999; Kühlmann et al., 2000), and between Asp63 of Im7 and Arg73 and Asn74 of the E7 DNase in the Im7–E7 DNase structure (Ko et al., 1999). However, the difference is consistent with the observation that loop 2 adopts a different conformation in the NMR structure of bound Im9 compared to the X-ray structure of the complex, and ties in with the finding that mutations in this region, Asp60Ala and Ser63Ala, do not significantly affect Im9 binding to the E9 DNase (Wallis et al., 1998).

Im9 binding and the slow conformational interchange of the DNase

Whittaker et al. (2000) have shown from NMR studies of isotopically labeled DNase in complex with unlabeled Im9 that the slow exchange between the two different conformers of the DNase persists in the interprotein complex, although Im9 binding does affect the dynamic behavior. In the free DNase, the rate of exchange from the major to the minor form is $1.61 \pm 0.11 \text{ s}^{-1}$ at 288 K and in the bound DNase it is $1.1 \pm 0.2 \text{ s}^{-1}$ at 298 K. The main regions of the DNase involved in the conformational exchange are around residues 20–25 and 67–72 (see Fig. 2 of Whittaker et al., 2000), with the dynamic properties of the latter being reduced by Im9 binding. The NMR data presented here together with that reported for the DNase by Whittaker et al. (2000) and the Im9–E9 DNase X-ray structure (Kleanthous et al., 1999) suggest a mechanism for the reduction in DNase dynamics.

The side chain of Asn72 of the E9 DNase is connected via a hydrogen bond to the backbone CO of Ile53 of Im9 in the complex (Fig. 8). As described above, the side-chain resonances of Ile53

Fig. 8. Ribbon diagram (generated in MOLMOL, Koradi et al., 1996) of the X-ray structure of the Im9–E9 DNase complex showing the interaction between Ile53 (cyan) of Im9 (yellow) and Asn72 (light grey) of the E9 DNase (red), and the connection between this and the side chain of Trp22 (see text).

show direct NOE contacts to the DNase in the complex consistent with hydrophobic interactions between Ile53 and its surrounding residues in the free protein being replaced by hydrophobic interactions with the DNase. The NOESY resonances of Ile53 in the complex are relatively strong compared to most other interface resonances and the Asn72 backbone NH of the DNase shows no evidence of dynamic exchange between the different DNase conformers in the complex, unlike the situation in the free state. Thus, the interaction between the Ile53 backbone of Im9 and the Asn72 side chain of the DNase anchors both residues and stabilizes one conformer of the DNase. However, the backbone NH peak of Asn70 of the bound DNase does show chemical exchange signals between two DNase conformers, though the chemical shift difference between the two conformers is significantly less than for the free DNase (see Fig. 2 of Whittaker et al., 2000). This indicates that the stabilization achieved by the contact between Ile53 of Im9 and Asn72 of the DNase only reduces the mobility of the 67–72 region of the DNase and does not abolish it. This region of the DNase is in direct contact with the side chain of Trp22 (Fig. 8), which is a central group in the DNase conformational heterogeneity since all NH groups of the free DNase within a 9 Å radius of any point of the Trp22 ring have multiple NMR peaks (Whittaker et al., 2000). Thus, Im9 binding to the DNase affects the 67–72 region through its contact with Asn72 of the DNase, leading to a reduction in the dynamic behaviour of the 67–72 region and an alteration in the environment of Trp22.

Materials and methods

Sample preparation

Unlabeled metal-free E9 DNase domain and uniformly [U -98% ^{13}C , U -98% ^{15}N]-labeled Im9 were prepared as described previously (Osborne et al., 1994; Wallis et al., 1994). The final concentrations of 1.2 mM ^{13}C , ^{15}N -labeled Im9 and 1.3 mM E9 DNase (in 20 mM phosphate buffer, pH 6.2, 90% H_2O /10% D_2O , 0.01% NaN_3) ensured that the Im9 was fully bound.

NMR spectroscopy

NMR spectra were obtained at 295 K with Varian Unity+, Varian Unity Inova and Bruker AMX spectrometers operating at 500, 600, and 750 MHz, all equipped with actively shielded z gradient 5 mm triple resonance probes. Acquisition parameters for the 3D experiments are summarized in Table 1 of Supplementary material in the Electronic Appendix. ^1H chemical shifts are listed relative to internal DSS while ^{13}C and ^{15}N chemical shifts are referenced indirectly to DSS as described by Wishart et al. (1995). All spectra were processed with NMRPipe (Delaglio et al., 1995) and analyzed in XEASY (Bartels et al., 1995). The acquired data were zero-filled to double the number of data points after the indirect dimensions were subject to one round of linear prediction to improve spectral resolution. The window function used was a squared sinebell, shifted by $\pi/2$ in all cases.

Structure constraints

NOEs were extracted from ^{13}C - and ^{15}N -edited 3D NOESY spectra acquired with ^{13}C decoupling during acquisition and with mixing times of 100 ms; 2,375 and 1,041 assigned cross peaks were

obtained, respectively. To identify intermolecular contacts between Im9 and E9 DNase, an additional ^{13}C -edited NOESY spectrum was acquired under identical conditions to the first one, but without ^{13}C decoupling during acquisition.

Qualitative NH exchange data were extracted from a series of sensitivity-enhanced ^1H , ^{15}N -HSQC (Kay et al., 1992) spectra acquired at different time intervals after dissolving the freeze-dried complex in 100% D_2O . The final spectrum of the series after 11 h was used to determine slowly exchanging amide protons.

Structure calculations

Distance restraints were derived from NOE cross peaks in ^{13}C - and ^{15}N -edited NOESY spectra via the automatic peak integration routine in XEASY. Peak volumes were converted into upper distance limits using a semi-automated calibration procedure in DYANA (Güntert et al., 1997). For the ^{15}N -edited NOESY data and the backbone peaks in the ^{13}C -NOESY spectrum, the volume of the $\alpha, N(i, i + 3)$ cross peak in an α -helix was used to calibrate the NOEs while the peaks involving side chains and methyl groups from the ^{13}C -edited NOESY spectrum were calibrated via the distance between the two H_γ protons in Glu residues and the H_β - CH_3 distance in Val, respectively. To take account of spin diffusion, both intramolecular and across the interface loose intensity calibrations were used (e.g., $\alpha, N(i, i + 3)$ distances were taken to be 3.7 instead of 3.4 Å). It was not possible to include any intermolecular NOEs because side-chain assignments for the DNase are incomplete. Inclusion of hydrogen bonds derived from the NH exchange data for established α -helical regions where preliminary structure calculations indicated the consistent presence of a hydrogen bond improved the consistency of the secondary structure elements and the RMSDs. However, incorporating backbone dihedral angles extracted from C_α and C_β chemical shifts (Wang & Bax, 1996) did not lead to any improvement of RMSDs.

Four hundred structures were calculated using the torsion angle dynamics routine in DYANA with the standard protocol performing 100 initial minimization steps followed by 800 steps of simulated annealing at the starting temperature of $T = 8.0$, where T is defined as the DYANA target function units per degree of freedom. After 3,200 steps of slow cooling to $T = 0$, the calculations finished with 100 final minimization steps and 200 steps of molecular dynamics. The 20 best structures of these 400 with no consistent NOE violations >0.5 Å and no angle violations >58 were subsequently subjected to a restrained molecular dynamics minimization in OPAL (Luginbühl et al., 1996) to eliminate residual van der Waals contacts. The 20 final structures were subjected to integrity checks using the programs AQUA and PROCHECK-NMR (Laskowski et al., 1996). All structure comparisons have been performed using the program MOLMOL (Koradi et al., 1996). Whenever side-chain RMSDs were calculated this was always based on a best fit for the backbone.

Structure analyses

Analyses of the 2.06 Å X-ray structure of the Im9–E9 DNase complex (Kleanthous et al., 1999) to obtain interatomic distances were performed using MOLMOL, while the program Total (Williamson & Asakura, 1993; Asakura et al., 1995) was used to calculate ^1H chemical shifts based on the X-ray structure of the complex.

Supplementary material in the Electronic Appendix

Table 1 includes experimental details for the NMR spectra.

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