Slow conformational dynamics of an endonuclease persist in its complex with its natural protein inhibitor

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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 isolated DNase domain uniformly labeled with ¹³C/¹⁵N bound to unlabeled Im9 contain more signals than expected for a single DNase conformer, consistent with the bound DNase being present in more than one form. The presence of chemical exchange cross peaks in 750 MHz ¹⁵N-¹H-¹⁵N HSQC-NOESY-HSQC spectra for backbone NH groups of Asp20, Lys21, Trp22, Leu23, Lys69, and Asn70 showed that the bound DNase was in dynamic exchange. The rate of exchange from the major to the minor form was determined to be $1.1 \pm 0.2 \text{ s}^{-1}$ at 298 K. Previous NMR studies have shown that the free DNase interchanges between two conformers with a forward rate constant of $1.61 \pm 0.11 \text{ s}^{-1}$ at 288 K, and that the bound Im9 is fixed in one conformation. The NMR studies of the bound DNase show that Im9 binds similarly to both conformers of the DNase and that the buried Trp22 is involved in the dynamic process. For the free DNase, all NH groups within a 9 Å radius of any point of the Trp22 ring exhibit heterogeneity suggesting that a rearrangement of the position of this side chain is connected with the conformational interchange. The possible functional significance of this feature of the DNase is discussed.

Keywords: chemical exchange; colicin E9; endonucleases; NMR

Colicin E9 (ColE9) is a plasmid-encoded DNase that is secreted as part of the stress response system of *Escherichia coli* to kill other bacteria (James et al., 1992, 1996). The toxic DNase activity of colicin E9 is contained in its 134 amino acid C-terminal domain (Wallis et al., 1994; Garinot-Schneider et al., 1996), with other domains being responsible for the toxin entering sensitive target cells via interaction with the extracellular receptor for vitamin B₁₂, BtuB (Di Masi et al., 1973; Taylor et al., 1998). To provide immunity against the action of the cytotoxic domain of ColE9, producing cells co-express a 9.5 kDa inhibitor protein, Im9, that binds to both the intact ColE9 and the separate, 14.5 kDa, E9 DNase domain with a K_d of $\sim 0.1 \text{ fM}$ (Wallis et al., 1995; Kleanthous

et al., 1998). Recent X-ray crystal structures have been reported for the E9 DNase-Im9 complex (Kleanthous et al., 1999) and the related colicin E7 DNase-Im7 complex (Ko et al., 1999) that reveal that the inhibitor protein binds to a region of the DNase far from the active site with its four helices adopting a “V-clamp” structure in its contact with the DNase. Inhibition of the DNase could come about from the bound inhibitor sterically interfering with binding of substrate DNA (Kleanthous et al., 1999) and/or from Im binding stabilizing an inactive conformation of the DNase (Whittaker et al., 1998). This latter possibility is suggested by the NMR detection of two slowly interconverting forms of the unbound E9 DNase in which the environment of a tryptophan was altered (Whittaker et al., 1998). The forward (k_F) and backward (k_B) rates of the NMR-detected interconversion ($k_F = 1.61 \pm 0.5 \text{ s}^{-1}$ and $k_B = 1.05 \pm 0.5 \text{ s}^{-1}$ at 288 K) are similar to the sum of the rates ($k_F + k_B = 4.4 \pm 0.4 \text{ s}^{-1}$ at 298 K) for a conformation change that accompanies Im9 binding to the E9 DNase observed in stopped-flow tryptophan fluorescence experiments (Wallis et al., 1995). It is not possible from the X-ray structures of the Im-DNase complexes alone to decide whether alterations to the dynamic properties of the DNase on inhibitor binding contribute to the inactivation of the DNase, and therefore, we have investigated the colicin E9

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; Col, colicin; ColE9, colicin E9; CSI, chemical shift index; E9 DNase, the expressed C-terminal 134 amino acids of colicin E9 encompassing the DNase activity; EXSY, chemical exchange spectroscopy; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ppm, parts per million; TOCSY, total correlation spectroscopy.

DNase-Im9 complex by NMR. Here we report that the complexed DNase interchanges slowly between different conformations while the bound inhibitor protein remains fixed in one.

Results and discussion

The bound DNase has two conformations in dynamic exchange

Sequence-specific assignments for backbone and side-chain atoms of the $^{13}\text{C}/^{15}\text{N}$ labeled E9 DNase in complex with unlabeled Im9 were obtained from HNCO, HNCA, CBCA(CO)NH, and HBHA(CBCACO)NH spectra (Grzesiek & Bax, 1992a, 1992b; Muthandiram & Kay, 1994). There were far more peaks than anticipated for a single conformation in all NMR spectra, as shown by the ^1H - ^{15}N HSQC spectrum (Fig. 1), indicating that the bound DNase was present in more than one form. Despite the spectroscopic complexity introduced by this structural heterogeneity, we have been able to assign the majority of the backbone resonances and many of the side-chain resonances of the two conformations. Analysis of the backbone chemical shifts using the chemical shift index (CSI) (Wishart & Sykes, 1994) indicates that the secondary structure of the bound E9 DNase detected by NMR agrees well with that for the X-ray structure (Kleanthous et al., 1999): α -helical

regions indicated by the CSI (and X-ray) involve residues 36–41 (36–42), 49–62 (50–62), 76–80 (75–80), and 125–130 (124–129), and β -sheet structures involve residues 100–102 (100–103) and 118–123 (119–122).

Twenty-eight backbone NH groups were found to have different ^1H and/or ^{15}N chemical shifts for the two conformers (Fig. 2A,B). The maximum chemical shift differences between the two conformations were found for the 20s region; for example, the peptide N and NH resonances of Leu23 shift by 5.9 and 0.86 ppm between the two conformers of the bound DNase. Some of the conformational heterogeneity involves regions in regular secondary structure, such as the 20s and late 30s regions (Fig. 2D).

To investigate the rate of exchange, we measured 750 MHz ^{15}N - ^1H - ^{15}N HSQC-NOESY-HSQC spectra (Frenkiel et al., 1990) at 298 K with different mixing times. Chemical exchange cross peaks were observed for backbone NH groups of Asp20, Lys21, Trp22, Leu23, Lys69, and Asn70 (Fig. 3A), confirming that the conformations of the bound DNase are in dynamic exchange. However, only the cross peak for Asn70 was sufficiently resolved to allow determination of the exchange rate from the major to the minor form (Fig. 3B). At $1.1 \pm 0.2 \text{ s}^{-1}$ (298 K) this is similar to the rate of interchange of the free DNase at 288 K. The equilibrium constant defined by the Asn70 diagonal peaks is ~ 1.2 , little altered from the 1.5 seen for the DNase (Whittaker et al., 1998). Thus,

Fig. 2. Normalized weighted average chemical shift differences, $\Delta_{av}/\Delta_{max}(H_NN)$, between the two conformers of the (A) free and (B) bound DNase plotted against residue number for the amide proton and nitrogen resonances. $\Delta_{av}(H_NN)$ was calculated using $\Delta_{av}(H_NN) = [(\Delta H^2 + (\Delta N/5)^2)/2]^{1/2}$, where ΔH and ΔN are the differences in chemical shift between the two conformers (Garrett et al., 1997). Assignments for the conformers of the free DNase have been reported previously (Whittaker et al., 1999) and deposited with BioMagResBank as entry 4293. Assignments for the bound DNase have been deposited with BioMagResBank as entry 4352. **C:** Backbone amide chemical shift perturbations of the major conformer of the DNase resulting from Im9 binding. Note that residues 8, 17, 33, 35, 65, 73, 85, 88, 106, and 124 are prolines and that the NH resonances of Met1, Glu2, Ala26, Lys89, and Thr123 are unassigned. **D:** Secondary structure of the bound DNase from the X-ray structure (Kleanthous et al., 1999).

Im9 binding does not appear to substantially disturb the DNase conformational equilibrium.

Im9 binds similarly to both conformers of the DNase

The previously determined DNase binding site on Im9 mapped by ^1H - ^{15}N chemical shift perturbations (Osborne et al., 1997; Boetzel

et al., 1998) agrees well with the binding region determined by X-ray crystallography (Kleanthous et al., 1999) and site-directed mutagenesis (Wallis et al., 1998). Only one set of resonances was detected for the bound Im9 (Osborne et al., 1997; Boetzel et al., 1998), indicating that it has only one conformation, and suggesting it is in contact with only one binding surface on the DNase. The reverse chemical shift mapping of the Im9 binding site on the

Fig. 3. A: Mixing time dependence of the ^{15}N - ^1H - ^{15}N HSQC-NOESY-HSQC spectrum of Im9-bound E9 DNase showing the chemical exchange cross peak for the backbone NH of Asn70 (labeled E) and the sequential NH-NH NOEs for Lys69 and Leu71. **B:** Build-up and decay curves for the chemical exchange cross peak (corresponding to exchange from the major to the minor conformer) and diagonal peaks of the NH of Asn70. Mole fractions for the major (x_A) and minor (x_B) forms were determined as the diagonal peak volume at $\tau_m = 0$: x_A , 0.54 and x_B , 0.46. Curve fitting the data to Equation 4 of Whittaker et al. (1998) resulted in the rate of exchange for the conversion of the major form into the minor form being $1.1 \pm 0.2 \text{ s}^{-1}$ with a relaxation rate R_1 of $6.3 \pm 1.0 \text{ s}^{-1}$.

DNase (Fig. 2C) also agrees well with the binding region determined by X-ray crystallography (Kleanthous et al., 1999). Furthermore, there is only one set of resonances for the bound DNase

in the Im9 binding site, although heterogeneity involving residues bordering the Im9 binding site is still present, i.e., residues 69–71 (Fig. 2B,C). This is consistent with the relatively small degree of

heterogeneity seen in the Im9 binding site for the free DNase (Fig. 2A) being removed by Im9 binding.

Mapping the dynamics in solution onto the X-ray structure

Many of the residues of the DNase taking part in the conformational exchange are clustered together, with both internal residues and residues located on the surface affected (Fig. 4). Though most of the solution studies have concerned metal-free DNase and the X-ray studies metal-containing DNase, ^1H - ^{15}N NMR studies of Zn^{2+} -containing DNase show that Zn^{2+} binding is not accompanied by substantial conformational changes (Hannan et al., 2000) and, importantly, the dynamic conformational equilibrium between the two DNase conformers is maintained (S.B.-M. Whittaker & G.R. Moore, unpubl. data).

The proposed DNA binding site with the transition metal ion at its center (colored green) is not much affected by the heterogeneity in either the free or Im9-bound DNase (Fig. 4). Nor is the heterogeneity much affected by the presence or absence of the bound metal ion. The relatively small degree of heterogeneity at the Im9 binding site is removed by complex formation, and there is a general decrease in the extent of heterogeneity elsewhere in the structure on Im9 binding. However, it is striking that even in the Im9-bound form the heterogeneity affects residues forming 17% of the solvent accessible molecular surface. What is not clear from the chemical shift analysis (Fig. 2A,B) is whether a group exhibits heterogeneity because it undergoes motion itself or because groups in its neighborhood are dynamic. However, an analysis of the

X-ray crystallographic *B*-factors for the E9 DNase-Im9 complex (Kühlmann, 1998) showing that relatively high *B*-factors are seen for residues 20 and 70, suggests that at least these groups are mobile. As with the observation of chemical shift heterogeneity (Fig. 2), some of the regions with higher than average *B*-factors include residues in α -helices.

Comparison of the X-ray structures of the E9 DNase-Im9 complex (Kleanthous et al., 1999) and the related colicin E7 DNase-Im7 complex (Ko et al., 1999) reveals an interesting feature that may be relevant for this study. The two DNase proteins have $\sim 72\%$ sequence identity, with the region from residue 20 to 29 having 70% sequence identity; the three amino acid differences in this region are all aspartates of the E9 DNase becoming asparagines in the E7 DNase. The backbone positions of the X-ray structures of the two DNases are virtually identical but a significant structural difference is that whereas residue 20 to 26 is helical in the E9 DNase it is a loop in the E7 DNase. Because asparagine has only a slightly smaller helix-forming tendency than aspartate (Creighton, 1993), this difference is unlikely to be due solely to the amino acid changes. It may be significant that the E7 DNase-Im7 complex was solved using X-ray data collected at room temperature, whereas the E9 DNase-Im9 complex was solved using data collected at 100 K, though our room temperature NMR experiments show the E9 DNase to be helical in this region in both its solution conformers. NMR studies of the E7 DNase are needed to investigate this further but whatever the cause of the structural difference is, its existence is consistent with this region not being fixed in one conformer for the E9 DNase.

Fig. 4. Structural representation of the regions of the DNase undergoing slow conformational exchange. **A:** Free DNase. **B:** DNase in complex with Im9. Red regions denote conformational heterogeneity. The metal ion displayed in green is included to help locate the active site. Each panel of **A** and **B** shows the DNase rotated anticlockwise about the *y*-axis through 0° , 90° , 180° , and 270° , respectively, with respect to the molecule in the left panel. The CPK representations were produced with MolMol (Koradi et al., 1996). The figure takes the structure of the DNase from the E9 DNase-Im9 X-ray structure to represent the structure of the DNase in solution both bound to Im9 and unbound. This is supported by the identification of the secondary structure and Im9 binding site of the DNase by NMR.

Fig. 5. Stereo representation of the X-ray structure (Kleanthous et al., 1999) of the bound DNase showing the relationship of the heterogeneous residues of the free DNase (colored red) to Trp22. For clarity, the entire backbone of the molecule is not shown. The figure was produced with MolMol (Koradi et al., 1996).

A buried tryptophan is associated with the slow dynamics

Trp22 is involved with the slow dynamics. There are two side-chain and backbone NH resonances of Trp22 for both the free and Im9-bound DNase, and all NH groups of the free DNase within a 9 Å radius of any point of the Trp22 ring (Fig. 5) exhibit heterogeneity. Furthermore, tryptophan fluorescence of the DNase is perturbed by Im9 binding in a biphasic manner (Wallis et al., 1995), with one of the phases having a slow rate similar to that detected in the NMR experiments (Whittaker et al., 1998). There is precedence in the protein NMR literature for mobility of tryptophan side chains to affect NMR spectra. An early example is afforded by Trp33 of tuna cytochrome *c*. The line widths of ^1H resonances of its side chain indicate that the ring reorients around its $\text{C}\alpha\text{-C}\beta$ and/or $\text{C}\beta\text{-C}\gamma$ bond(s) (Moore & Williams, 1980). The spectroscopic characteristics we have described here for Trp22 of the DNase are similar to those seen by Sørensen et al. (1996) in their NMR study of a collagen Kunitz domain. The side chain of Trp21 of the collagen domain reorients between two conformations with a rate constant of $\sim 1\text{ s}^{-1}$, and the tryptophan movement causes multiple resonances from groups in the central β -sheet of the Kunitz domain. Resonances of Leu23 of the E9 DNase are shifted upfield by Trp22, and their temperature-dependent behavior (Fig. 6) suggests that the spatial relationship of Leu23 with respect to Trp22 changes with temperature consistent with a temperature-dependent movement of Trp22 about its $\text{C}\alpha\text{-C}\beta$ and/or $\text{C}\beta\text{-C}\gamma$ bond(s), though this may only be a small structural change.

$^1\text{H}\text{-}^1\text{H}$ slices of $^1\text{H}\text{-}^{15}\text{N}$ NOESY-HSQC spectra for the two Trp22 indole NH resonances of the free DNase show they have different NOE connectivities (Fig. 7). Full side-chain assignments have not been obtained, and so it is not possible to locate unambiguously the position of the Trp22 ring in both conformers of the DNase. However, it is clear that the indole ring is relatively close to Leu23 and Leu67 in one or more of its conformations. The situation is complex, however, because the $^1\text{H}\text{-}^1\text{H}$ slice of the $^1\text{H}\text{-}^{15}\text{N}$ NOESY-HSQC spectra for the Trp22 indole NH at 10.55 ppm (^{15}N , 130.7 ppm) contains connectivities to both Leu23 and Leu67 (Fig. 7). Leu67 is on the opposite side of Trp22 from

Leu23, and without a rearrangement of the Trp22, Leu23, and/or Leu67 side chains from their positions seen in the X-ray structure it is not possible to bring them together to obtain the NOE con-

Fig. 6. Upfield region of the ^1H -NMR spectrum of unbound Zn^{2+} -containing E9 DNase at varying temperatures. Labeled resonances from Leu23 were assigned from TOCSY and NOESY spectra as described in the caption to Figure 7.

Fig. 7. ^1H - ^1H slices of a 600 MHz ^1H - ^1H - ^{15}N NOESY-HSQC spectrum ($\tau_m = 100$ ms) of Zn^{2+} -containing E9 DNase. The slices were taken at the amide proton and nitrogen chemical shifts of the residues labeled at the top of each slice. For the indole NH of Trp22, a and b identify the two conformers; peaks labeled E are chemical exchange cross peaks; horizontal lines indicate NOE connectivities from the indole NH of one conformer to side-chain protons of Leu23 and Leu67. $\gamma\text{CH}-\delta\text{CH}_3$ connectivities were obtained from 2D ^1H - ^1H TOCSY spectra ($\tau_m = 75$ ms). TOCSY connectivities from the NH resonances were not obtained for the full leucine side chains, but NOESY connectivities were observed in ^1H - ^1H - ^{15}N NOESY-HSQC spectra between the resonances of the $\gamma\text{CH}-\delta\text{CH}_3$ spin-systems and their intrasidic backbone NH resonances, which had been assigned using sequential triple-resonance spectra. The TOCSY data established the spin systems to be $\text{CH}-\text{CH}_3$ units of leucine or valine residues, and the intrasidic NOESY data indicated the assignments given. Furthermore, without a substantially greater difference between the X-ray structure and the solution structure than referred to in the text the δCH_3 resonances cannot be assigned in any other way.

nectivities observed. Thus, the indication from this is that the E9 DNase in solution adopts at least two conformations, neither of which are identical to the conformation seen in the X-ray structure. Even if the leucine side-chain resonance assignments are incorrect, there has to be a difference between the solution and solid-state

structures in the region of Trp22. The TOCSY data established the spin systems to be $\text{CH}-\text{CH}_3$ units of leucine or valine residues, and intrasidic NOESY data indicated the assignments given. The X-ray structure shows that there are only four $\beta/\gamma\text{CH}-\gamma/\delta\text{CH}_3$ spin systems within 10 Å of the Trp22 indole NH: Val18, Leu23, Leu67, and Val121. Resonances of the $\beta\text{CH}-\gamma\text{CH}_3$ spin systems of Val18 and Val121 have been assigned from ^1H - ^1H - ^{15}N TOCSY-HSQC and ^1H - ^1H - ^{15}N NOESY-HSQC spectra, with both through-bond and through-space connectivities linking their backbone NH resonances with the $\beta\text{CH}-\gamma\text{CH}_3$ resonances. Thus, for the $\beta/\gamma\text{CH}-\gamma/\delta\text{CH}_3$ spin systems indicated as Leu23 and Leu67 in Figure 7 to arise from other amino acids requires a substantial difference to the X-ray structure.

Do the slow dynamics have a functional significance?

Because the DNase-Im complex has to be dissociated to allow the nuclease action to take place, we proposed (Whittaker et al., 1998) that the DNase conformational heterogeneity might play a role in this. Scheme 1, for which $K_n = k_n/k_{-n}$ and $K_1K_2 = K_3K_4$, describes the behavior of the E9 DNase domain. Free DNase exists in conformations N_A and N_B , and if Im9 only bound to N_B (i.e., $K_3 \gg K_4$, $K_3 \gg K_1$, and $K_2 \gg K_4$), then receptor binding of the DNase-Im complex might force the dissociation of the complex if it had a strong preference for binding N_A over N_B .

Such a proposal is consistent with NMR studies of the free DNase (Whittaker et al., 1998), stopped-flow fluorescence studies of Im binding to the DNase (Wallis et al., 1995), and the single conformer observed for the X-ray structure of the complex (Kleantous et al., 1999). However, the NMR studies reported here showing the coexistence of $N_A I$ and $N_B I$ indicate that this model is incorrect. Nevertheless, the stopped-flow fluorescence studies show that Im9 binding and the reorientation of Trp22 are coupled events. Because the passage of the DNase through membranes may involve some degree of unfolding (Pommer et al., 1999), the conformational interconversion involving Trp22 may be coupled to more than just inhibitor protein binding. Thus, the properties of species such as $\text{BtuB}-N_A$, $\text{BtuB}-N_A I$, membrane- N_A , and membrane- $N_A I$, and their N_B counterparts needs to be investigated.

Methods

Sample preparation

Uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled E9 DNase in complex with unlabeled Im9 was prepared by adding solid unlabeled Im9 (freeze-dried from 90% $\text{H}_2\text{O}/10\%$ D_2O , potassium phosphate buffer, pH 6.2) to metal-free (Hannan et al., 1999), uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled E9 DNase (Whittaker et al., 1998) in solution (potassium phosphate buffer, pH 6.2, 90% $\text{H}_2\text{O}/10\%$ D_2O). Zn^{2+} -containing ^{15}N -E9 DNase was prepared by the addition of stoichiometric amounts of ZnCl_2 to metal-free ^{15}N -E9 DNase. Final protein concentrations were ~ 2 mM E9 DNase and ~ 2.2 mM Im9 to ensure that the DNase was fully bound. This was confirmed by gel filtration.

NMR spectroscopy

NMR experiments were recorded at the SON NMR Large-Scale Facility in Utrecht on Bruker DMX-600, Varian Unity INOVA 500, and Varian Unity INOVA 750 spectrometers, and at UEA on a Varian Unity INOVA 600 spectrometer. 3D HNCO, HNCA, CBCA(CO)NH, and HBHA(CBCACO)NH spectra (Grzesiek & Bax, 1992a, 1992b; Muhandiram & Kay, 1994) were acquired using standard pulse sequences. 3D ^{15}N - ^1H - ^{15}N HSQC-NOESY-HSQC spectra (Frenkiel et al., 1990) were recorded on the 750 MHz spectrometer. 2D ^1H - ^{15}N HSQC, 3D ^1H - ^1H - ^{15}N NOESY-HSQC ($\tau_m = 100$ ms) and 3D ^1H - ^1H - ^{15}N TOCSY-HSQC ($\tau_m = 75$ ms) spectra were recorded on the Varian 600 MHz spectrometer using pulse sequences in the Varian "Protein Pack" suite. All data were processed on Silicon Graphics Indy/O2 workstations using NMRPipe (Delaglio et al., 1995). Proton chemical shifts were referenced relative to the water signal. Carbon and nitrogen chemical shifts were calculated using the ratio of gyromagnetic ratios ($\gamma_{\text{C}}/\gamma_{\text{H}} = 0.251449530$; $\gamma_{\text{N}}/\gamma_{\text{H}} = 0.101329118$). Spectra were analyzed using FELIX 95.0 (Biosym/MSI, San Diego, California) or XEASY (Bartels et al., 1995). The exchange rate was determined as previously described by Whittaker et al. (1998), using XEASY (Bartels et al., 1995) for peak volume integrations. Data were curve fitted using the graphics software ORIGIN 4.1 (Microcal Software, Inc., Northampton, Massachusetts).

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