Structural inhibition of the colicin D tRNase by the tRNA-mimicking immunity protein

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Colicins are toxins secreted by *Escherichia coli* in order to kill their competitors. Colicin D is a 75 kDa protein that consists of a translocation domain, a receptor-binding domain and a cytotoxic domain, which specifically cleaves the anticodon loop of all four tRNA^{Arg} isoacceptors, thereby inactivating protein synthesis and leading to cell death. Here we report the 2.0 Å resolution crystal structure of the complex between the toxic domain and its immunity protein ImmD. Neither component shows structural homology to known RNases or their inhibitors. In contrast to other characterized colicin nuclease–Imm complexes, the colicin D active site pocket is completely blocked by ImmD, which, by bringing a negatively charged cluster in opposition to a positively charged cluster on the surface of colicin D, appears to mimic the tRNA substrate backbone. Site-directed mutations affecting either the catalytic domain or the ImmD protein have led to the identification of the residues vital for catalytic activity and for the tight colicin D/ImmD interaction that inhibits colicin D toxicity and tRNase catalytic activity.

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Introduction

The plasmid-encoded antibacterial toxins called colicins are present in many natural strains of *Escherichia coli*, and secreted into the extracellular medium under a variety of environmental stress conditions. These highly efficient and specific proteins kill competing bacteria that are not able to produce the neutralizing immunity protein (Imm), usually co-synthesized by colicinogenic cells. In the case of nuclease colicins, which target hydrolysable substrates in the cytoplasm, the colicin producer cells are protected against both endogenous and exogenous toxin molecules by forming a tight 1:1-heterodimer complex with the cognate Imm protein (Pugsley, 1984; Braun *et al.*, 1994; James *et al.*, 1996; Kleanthous *et al.*, 1998).

The lethal invasion of the target cell proceeds through three distinct steps: receptor recognition and binding, translocation across both the outer and inner membranes and cytotoxicity. Most colicins display a similar tripartite architecture of functional domains (Lazdunski *et al.*, 1998), each of which is related to a specific step of penetration or killing activity. The central R region is necessary for interacting with the cell surface receptor, whereas the subsequent penetration of the outer cell membrane requires the N-terminal T (translocation) domain. The cytotoxic activity is associated with the 10–15 kDa C-terminal part of the colicin molecule. E-type nuclease colicins bind to the outer membrane BtuB receptor, usually involved in the high-affinity uptake of vitamin B₁₂, and require the outer membrane protein OmpF and the TolA–B–Q–R system for their import (Lazdunski *et al.*, 2000; Bouveret *et al.*, 2002; James *et al.*, 2002; Lazzaroni *et al.*, 2002). The immunity protein is released from the naturally secreted colicin–Imm complex when it is adsorbed to the target cell surface (Krone *et al.*, 1986).

In contrast to E-type colicins, colicins D and B bind to the outer membrane receptor FepA, necessary for the internalization of ferric enterobactin, a siderophore, and penetrate target bacteria by appropriating the multi-protein system of the host cell required for Fe³⁺ uptake, namely the energy-dependent TonB/ExbB–D pathway (Braun *et al.*, 2002; Ferguson and Deisenhofer, 2002; Postle and Kadner, 2003). The first 313 N-terminal amino acids of colicins B and D, sufficient for their translocation across the outer membrane, are 96% identical (Roos *et al.*, 1989). In contrast, the C-terminal regions carrying the killing function are unrelated, as *de facto* colicin B has pore-forming lethal activity rather than nucleolytic activity (Pressler *et al.*, 1986). In the colicin B polypeptide, the common T and R domains are immediately followed by the killing pore-forming domain, whereas in colicin D a linker region of 280 residues, with no known function, separates the import and the catalytic domains (de Zamaroczy and Buckingham, 2002). The small C-terminal catalytic domain of colicin D, 91 amino acids long and starting at Lys607, has been shown to be necessary and sufficient for full enzymatic activity (de Zamaroczy *et al.*, 2001). In the case of colicin D, the inner membrane leader peptidase LepB was shown to be an essential and specific requirement for cell killing (de Zamaroczy *et al.*, 2001). Although LepB is necessary for colicin D proteolysis *in vitro*, its precise role remains unknown.

Target cell death results from either a nonspecific DNase activity (colicins E2, E7, E8 and E9; Toba *et al.*, 1988; Eaton and James, 1989; Chak *et al.*, 1991) or a highly specific RNase activity. Among these latter, colicins E3, E4, E6 and the closely related cloacin DF13 are ribosomal endonucleases, referred to as ribotoxins, whose catalytic activity results in a specific nucleolytic break of the 16S ribosomal RNA (Bowman *et al.*, 1971; de Graaf *et al.*, 1973; Lasater *et al.*, 1989). Colicins E5 and D hydrolyse different classes of transfer RNAs. The first specifically cleaves tRNAs in which

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the guanine located at the wobble position is modified to queuine (Ogawa *et al*, 1999), whereas the second hydrolyses the four arginine isoacceptors at a nearby position in the anticodon loop, between nucleotides 38 and 39 (Tomita *et al*, 2000). RNase colicins thereby provoke cell death by inactivating the protein biosynthetic machinery.

Here, we show the crystal structure of colicin D transfer-RNase domain with its cognate immunity protein at 2 Å resolution. The components of the binary complex are structurally unrelated to previously described colicins, immunity proteins or RNases. Functionally important residues in both colicin D and ImmD have been identified by structure-based site-directed mutagenesis and analysed both *in vivo* and *in vitro*. The highly efficient mechanism by which ImmD inhibits the activity of the tRNase domain by binding directly to the active site is discussed in relation to observations concerning other nuclease–immunity protein complexes.

Results

Structure determination

Analysis of the solvent content of crystals obtained from the colicin D–ImmD complex revealed that these could not contain the entire colicin D complex, despite the fact that this was the starting protein material in the crystallization drops. SDS–PAGE and N-terminal peptide sequencing indicated that the crystallized protein material corresponded to a complex between the tRNase domain of colicin D and ImmD. The crystal structure of this complex was solved to 2.0 Å resolution by the Single wavelength Anomalous Dispersion (SAD) phasing method from a selenomethionine-labelled protein crystal containing one copy of the complex in the asymmetric unit. Well-defined electron density was observed for residues Leu591–Leu697 from colicin D, comprising the minimal, 91-amino-acid-long catalytic domain necessary and sufficient for lethal tRNase activity, which starts at Lys607 (de Zamaroczy *et al*, 2001), and all residues (Met1 to Leu87) from ImmD, as well as for 196 water molecules and a PEG 400 molecule from the crystallization conditions. The molecular masses of these fragments are in accordance with those determined by mass spectrometry. The refinement statistics are gathered in Table I.

Further biochemical analysis of the crystallization drops showed that colicin D is gradually proteolysed during the nucleation process, resulting in a resistant core defined by the complex between the catalytic domain and ImmD, the fragment that finally crystallized. Once this was understood, crystals could also be obtained by cloning and purifying only the catalytic domain of colicin D in complex with ImmD (data not shown).

Structure of colicin D and ImmD

The colicin D catalytic domain adopts a crescent-shaped globular α/β fold of approximate dimensions $25 \times 30 \times 40$ Å³ and is composed of a central four-stranded antiparallel β sheet (β 1, β 2, β 3 and β 4) sandwiched between a long helix (α 2) on one side and the C-terminal helix (α 3) on the other (Figure 1A). A third kinked helix (α 1) lies in the plane of the β sheet, interacting with strand β 4, perpendicular to helix α 2. Searches for structural homologues of the catalytic domain using the EBI Macromolecular Structure Database (<http://www.ebi.ac.uk/msd/>) revealed mediocre matches with known structures (best Z score obtained: 3.2).

Table I Data collection statistics

	Native	SAD
<i>Data collection</i>		
Resolution (Å)	99–1.98 (2.02–1.98)	25–3.0 (3.09–3.0)
Space group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Unit cell parameters	$a = b = 62$ Å; $c = 147.9$ Å	$a = b = 61.9$ Å; $c = 150$ Å
Total number of reflections	273269	231794
Total number of unique reflections	20818	11111
R_{sym} (%) ^a	5.8 (27.8)	11.5 (37.8)
Completeness (%)	98.3 (86.7)	100 (100)
$I/\sigma(I)$	18.9 (1.6)	20.2 (5.1)
Redundancy	13	21
<i>Phasing</i>		
Resolution (Å)		80–3.0
FoM (after density modification)		0.25 (0.55)
<i>Refinement</i>		
Resolution (Å)	20–2.0	
R/R_{free} (%) ^b	19.4/23.5	
R.m.s.d. bonds (Å)	0.005	
R.m.s.d. angles (deg)	1.07	
Mean B factor (Å ²)	27/34	
prot/water		
Ramachandran plot		
Most favoured	89%	
Allowed	11%	

Values in parentheses are for the highest resolution shell.

^a $R_{\text{sym}} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$, where I_{hi} is the i th observation of the reflection h , while $\langle I_h \rangle$ is the mean intensity of reflection h .

^b $R_{\text{factor}} = \sum ||F_o| - |F_c|| / |F_o|$. R_{free} was calculated with a small fraction (5%) of randomly selected reflections.

The colicin D immunity protein adopts an antiparallel four helical bundle fold of dimensions $20 \times 20 \times 40$ Å³ (Figure 1A). The helices are tightly packed, forming a compact cylindrical molecule. The first antiparallel helical pair α 1, α 2 packs against the second pair formed by helices α 3 and α 4, with an angle of about 30°. No significant structural analogies to other proteins have been found. The location of α helices and β sheets in the peptide sequence of the colicin D and ImmD is depicted in Figure 2A and B.

The colicin D tRNase active site

It has been shown that the smallest colicin D fragment capable of cleaving tRNA^{Arg} corresponds to the Lys607–Leu697 fragment (de Zamaroczy *et al*, 2001). The crystallized colicin D protein in this study contained residues 591–697 and therefore corresponds to a catalytically active fragment. Except for colicin E5, His residues play a crucial role in the catalytic mechanism of RNases. At least one His, consistently present in the active site, is directly involved in proton abstraction of the 2'-OH ribose proton of the RNA substrate (Fersht, 1999). Colicin D specifically cleaves tRNA^{Arg} isoacceptors between nucleotides 38 and 39, and mutation of His611 abolishes its cleavage and toxicity activities (Tomita *et al*, 2000). This His residue is located at the C-terminal end of the α 1 helix, which forms the rim of the central groove of the protein (Figure 1B). The bottom of the groove is rather hydrophobic (Leu676 and Trp679 from the β 4 sheet). His611 is further surrounded by two polar residues: Ser677 (strand β 4)

Figure 1 Stereo views. (A) Ribbon representation of the complex between colicin D (yellow) and ImmD (pink). The colicin D His611 and the ImmD Glu56 side chains at the interface are shown as sticks. Residues whose mutation has no effect on the *in vivo* and *in vitro* activities are highlighted in green. (B) Close-up of the colicin D putative active site. The colicin D tRNase domain is shown as a ribbon with the potential active site residues represented as sticks. For clarity, residues are identified by their one-letter code in all figures. (C) Close-up of main colicin D (yellow) and ImmD (green) residues involved at the dimer interface.

and Asp614 (on helix $\alpha 1$). The opposite rim of the groove carries charged and polar residues (Tyr649, Arg651, Glu652 and Lys670). Helix $\alpha 1$ further carries four Lys residues (Lys603, Lys607, Lys608 and Lys610), creating a positive charge cluster (Figures 1A and B) that could play an important role for substrate binding by neutralizing the negatively charged phosphate groups of the substrate tRNA backbone.

To clarify their functional involvement in tRNA hydrolysis and/or tRNA recognition, most of the residues mentioned above were changed into alanine and both the *in vitro* ribonuclease activity and the cytotoxicity of the mutants were compared to those of the wild-type protein (Table II). Northern blot analysis of tRNA^{Arg}CCG cleavage *in vitro* revealed that colicin molecules with mutated His611 or lysine residues forming the positive charge cluster (except for Lys603) are either inactive (Lys608, Lys610 and His611) or

Figure 2 Molecular surface representation. (A, B) Secondary structure assignments along the colicin D (A) and ImmD (B) sequences. Residues involved in complex formation are highlighted in bold. (C) The binary colicin D (yellow)–ImmD (green) complex. The orientation is the same as in Figure 1A. The red-coloured region corresponds to ImmD positions located outside the interface, but whose mutation abolishes or reduces the inhibitory capacity. (D) Surface mapping of the colicin D catalytic active site residues. Unmutated positions are coloured grey. Residues whose mutation does not alter tRNase activity are shown in green, those whose substitution results in partially or fully inactivated proteins are depicted in orange and red, respectively. For clarity, only residues whose mutation results in complete loss of activity are labelled. (E) ImmD foot-printing (green) at the colicin D surface. The His611 residue is coloured yellow. The orientation is the same as in (D). (F) Open leaflet representation of the complex illustrating the electrostatic complementarity between colicin D and ImmD. Regions of the surface with negative potential are coloured red and those with positive potential are in blue. The colicin D orientation is similar as in (D). The ImmD is rotated 180° around the vertical axis. (G) Surface mapping of the ImmD residues involved in the inhibitory effect. Colour coding is the same as in (D). The orientations of the ImmD in the top and bottom panels are related by a 180° rotation around the vertical axis.

only partially active (Lys607). Mutant tRNases affecting the main residues forming the central groove (Ser677, Trp679) or its rim (Arg651) are not active, while the Asp614Ala mutant colicin D that had activity *in vitro* was found to be seriously impaired in its *in vivo* toxicity (Figure 2D). Finally, mutation of residues located upstream of position 607 (for instance, Met590Ala, Leu591Ala, Asn592Ala (data not shown) and Leu595Pro, Ser601Pro, Arg602Gly, Lys603Glu, Asp606His (Table II)) did not affect the catalytic activity. This is in

Table II Site-directed mutagenesis^a

Mutated ColD residue	<i>In vivo</i> ^b		Mutated ImmD residue	<i>In vivo</i> ^b	
	colD + ImmD	<i>In vitro</i> ^c –ImmD + ImmD		colD + ImmD	colD + ImmD
wt ColD	+	+	wt ImmD	+	–
L595P	+	+	K11A	+	–
S601P	+	+	L12A	+	–
R602G	+	+	S16A	+	–
K603E	+	+	K17A	+	–
D606H	+	+	E22A	+	–
K607A	+ / – –	+ / – –	R26A	+	–
K608A	–	–	E30A	+	–
K610A	–	–	R31A	+	+
H611A	–	–	R32A	+	–
D614A	+ / – –	+	R32Q	+	+ / –
Y649A	+ / –	+ / –	R33A	+	–
R651A	–	–	Y35A	+	–
E652A	+	+	N46A	+	+ / –
K670A	+	+	E49A	+	+ / – –
S677A	–	–	F52A	+	+
W679A	–	–	M53A	+	–
K680A	+	+	E56A	+	+ / –
N682A	+ / –	+ / –	R57A	+	+ / – –
A685Q	+ / –	+	E59A	+	+
D686A	+	+ / –	D61A	+	–
			D63A	+	–
			Y67A	+	–
			E68A	+	–
			N72A	+	+ / –
			K75A	+	+
			V76A	+	+ / –
			R79A	+	+
			E83A	+	+ / –
			K84A	+	+ / –

Substitutions of functionally important residues are indicated by bold characters.

^aFull-sized wild-type or mutated colicin D molecules, with or without the wild-type or mutated ImmD protein are synthesized in a coupled *E. coli* S30 transcription/translation system (see Materials and methods).

^bThe cytotoxicity of the mutant proteins, spotted directly onto a lawn of sensitive wild-type cells, was determined by halo test.

agreement with previous experiments performed with truncated colicin D proteins constructed to determine the minimal tRNase domain (de Zamaroczy *et al*, 2001). These structure-based site-directed mutagenesis experiments indicate that the central groove and the positive charge cluster may form the active site of the colicin D catalytic domain.

The colicin D tRNase–ImmD complex

In the complex, ImmD fits smoothly in the central groove described above that potentially harbours the active site of colicin D (Figures 1A and 2C). One face of the ImmD helical bundle, formed by helices $\alpha 2$ and $\alpha 3$, interacts with residues from the bottom and both rims of the colicin D groove, comprising helix $\alpha 1$ and the strand $\beta 4$ –helix 3 segment. The orientation of these helices is parallel to helix $\alpha 1$ of colicin D. Most remarkably, ImmD seems to block completely the active site groove of colicin D (Figure 2D and E). Although the colicin D active site and the colicin D region responsible for ImmD binding seem to overlap, as demonstrated here, three residues were nevertheless identified, the mutation of which specifically affected only either catalytic activity (Tyr649, Asn682 and Asp686) or inhibitory interaction (Ala685) (Table II). This suggests that the regions of the colicin D catalytic domain involved in recognition and interaction of the tRNA^{Arg} and ImmD are not strictly identical.

Table III Salt bridges and hydrogen bonds involved in the ColD–ImmD complex

Colicin D	Distance (Å)	ImmD
Lys 607 N ζ	3.0	O ϵ 1 Glu 59
Lys 608 N ζ	3.1	O ϵ 2 Glu 56
Lys 610 O	2.9	N η 2 Arg 32
His 611 N ϵ 2	2.6	O ϵ 1 Glu 56
Asp 614 O δ 1	2.8	N ϵ Arg 32
Glu 652 O ϵ 1	2.6	O η Tyr 67
Ser 677 O γ	2.7	O ϵ 2 Glu 56
Lys 680 O	2.7	N η 1 Arg 31
Lys 680 O	2.8	N η 2 Arg 31
Ile 681 O	2.7	O η Tyr 35
Asp 686 N	2.8	O δ 1 Asn 46

The colicin D–ImmD complex formation buries a total protein surface area of 1920 Å² with approximately equal contributions from both molecules. This value is in the upper range of the average calculated for other stable protein–protein complexes (Lo Conte *et al*, 1999). Heterodimer formation involves 25 residues from the colicin D domain and 22 from the immunity protein (Figure 1C). The interface is stabilized by polar and electrostatic interactions: four salt bridges and seven hydrogen bonds were identified (Table III). The most prominent feature is a high degree of charge

complementarity between the positively charged patch on colicin D (Lys603, Lys607, Lys608, Lys610) and a corresponding negatively charged patch on ImmD (Glu56, Glu59, Asp61, Asp63, Glu68), resulting in three direct salt bridges (Figure 2F). Another salt bridge is contributed by Asp614 and Arg32 from ImmD. Most of the hydrogen bonds involved in complex formation occur between the main chain atoms from colicin D and side chains from ImmD (Table III).

In order to further probe the colicin D–ImmD interaction surface, the ImmD residues involved in complex formation were systematically mutated to alanine and the inhibitory effect of the mutant proteins on the tRNase activity and the toxicity of colicin D was compared to that of the wild-type immunity protein (Table II). This approach revealed that some aromatic and hydrophobic residues buried upon complex formation (Tyr35, Met53 and Tyr67) play a minor role in the interaction. In contrast, the change to alanine of most polar residues contributing to salt bridges and hydrogen bonds (Arg31, Asn46, Glu49 and Glu59), together with the Arg32Gln mutation (but not Arg32Ala), reduced or completely abolished the ImmD inhibitory capacity (Table II and Figure 2G). There are two more residues on the $\alpha 2$ helix whose mutation causes loss of inhibition of ImmD: Arg57 that stacks onto the guanidinium group of colicin Arg651, and Phe52 that is packed against colicin Trp679. Mutation of Glu56, which is in direct interaction with the colicin His611 in the complex structure, led to only 50% inhibition. Taken together, these biochemical and biological results support the importance of the polar interactions identified at the colicin D–ImmD interface and indicate that charge complementarity between the surfaces of both partners is a major factor in recognition.

The tRNase activity of several N-terminally truncated colicin D proteins, starting between positions 455 and 590, was shown to be resistant to inhibition by ImmD. However, the minimal tRNase domain itself was fully inhibited by the co-expressed immunity protein (de Zamaroczy *et al*, 2001). This suggests that ImmD might recognize more than one region of the whole colicin D molecule. In the case of ImmE3, the interaction with the complete colicin E3 was clearly shown to involve the globular translocation and the catalytic domains (Soelaiman *et al*, 2001). In order to identify an interaction surface of ImmD other than that in contact with the catalytic domain of colicin D, we have systematically mutated residues that are exposed to solvent and situated at the opposite side of the colicin D–ImmD interface presently observed in the crystal structure. Although substitution by Ala of residues Lys11, Leu12, Ser16, Lys17, Glu22, Arg26 and Glu30, from the $\alpha 1$ and the N terminal part of $\alpha 2$ helices did not affect the inhibitory action of ImmD, substitution of residues belonging to the $\alpha 4$ helix led to a complete (Lys75 and Arg79) or partial (Asn72, Val76, Glu83 and Lys84) loss of wild-type colicin D inhibition (Table II, Figure 2C and G). These observations raise the possibility that in the context of a complex between the intact colicin D–ImmD, the immunity protein is sandwiched between the catalytic and one or more N-terminal domains of colicin D.

Discussion

The studies we report here reveal that the structure of the catalytic tRNase domain of colicin D is unrelated to other

RNases. Similarly, ImmD is unlike previously studied RNase inhibitors. Our mutational studies have largely identified the amino-acid residues important both for the catalytic activity of the tRNase domain, and the interaction between this domain and ImmD that efficiently inhibits the enzymatic activity. Unlike other colicin–Imm complexes for which structures have been solved, ImmD completely blocks access of small molecules to the active site, and appears to present to the catalytic domain a pattern of negative charge that mimics the tRNA substrate.

Catalytic site and substrate recognition

The general catalytic mechanism by which ribonucleases hydrolyse RNA has been thoroughly studied by protein engineering and crystallographic analysis over several decades (Gilliland, 1997). RNase A, for instance, has two active site His residues that cooperate during the catalytic cycle (Findlay *et al*, 1961). One acts as a general base and abstracts a proton from the ribose 2'-OH, thereby catalysing the nucleophilic attack of this hydroxyl group on the 3'-phosphate group, leading to a cyclic intermediate, while the other serves as catalytic acid during the first cyclization step. Their catalytic roles are reversed during the subsequent hydrolysis of the cyclic intermediate. Other ribonucleases, such as barnase (Fersht, 1999) and colicin E3 (Zarivach *et al*, 2002), proceed probably through a similar mechanism, but with a His and a Glu as catalytic residues.

In the absence of any structural match with already known nuclease structures, it was not possible in the case of colicin D to predict catalytic or substrate binding residues. We therefore probed the potential active site region by systematic site-directed mutagenesis of residues situated in and around a depression on the surface of colicin D. Taken together, the results from the site-directed mutagenesis experiments show a clear picture of the catalytic pocket centred around His611 (Figure 2D). Substitution of this His by Ala or Tyr results in the loss of the catalytic activity, supporting the idea that His611 is the general base of the cyclization step of the reaction (Tomita *et al* (2000) and this work). From the structure, the only residue susceptible to act as a general acid seems to be Asp614, whose carboxylate group is at 4.5 Å from the His611 side chain. However, as the Asp614Ala mutant retains a significant though low *in vivo* cytotoxicity and a wild-type tRNase activity (Table II), it is very unlikely that this Asp plays a critical role in the tRNA hydrolysis reaction. Colicin E5, which targets the anticodons of tRNAs for Tyr, His, Asn and Asp, has no sequence analogy with colicin D even in the catalytic domain and lacks His residues altogether. Both tRNases cleave the tRNA anticodon loop, yielding a cyclic 2', 3'-phosphate and 5'-OH termini. The same is true for a further anticodon nuclease PrrC that provokes depletion of tRNA^{Lys} in T4 phage-infected *E. coli* cells (Levitz *et al*, 1990; Kaufmann, 2000). These observations suggest that there exist several active site solutions to cleave tRNA molecules. As the tRNA^{Arg} isoacceptors sensitive to colicin D yield 5'-half fragments with a 2',3'-cyclic phosphate group (Tomita *et al*, 2000), this tRNase apparently catalyses only the first cyclization step, releasing the cyclic product from the active site. This may explain why only a single His is present in the putative active site of colicin D and why a second catalytic residue seems to be absent.

The nucleophilic attack of the 2'-OH of the ribose on the 3'-phosphate group creates a negatively charged pentacovalent transition state that can be stabilized by nearby positively charged residues. In colicin D, the amine groups of Lys608 and Lys610 are well positioned to play such a role, as they are located 5.5 and 4.8 Å away, respectively, from His611. Their mutation to Ala abolishes both cytotoxic and RNase activities of colicin D, confirming their role in tRNA substrate recognition or more directly in the catalytic activity. Mutation of Ser677, whose hydroxyl group points towards His611 Nε2 (at 3.44 Å), abolishes both cytotoxic and RNase activity, suggesting a role for Ser677 in substrate binding and/or catalysis. Two explanations can be found for this result. First, in the absence of ImmD bound to colicin D, the His611 Nε2 atom could be within hydrogen bonding distance of the Ser677 hydroxyl group. As a result, one side of the His611 ring would be accessible to the substrate and oriented to act as a general base. Alternatively, Ser677 could be in direct interaction with the tRNA substrate and play a role in tRNA^{Arg} binding. A similar situation occurs in RNase A, where Thr45 is involved in pyrimidine specificity through its OH group (delCardayre and Raines, 1994). Ser677 is involved in both interactions with ImmD and RNase activity. Our assays do not allow the independent evaluation of these activities. Trp679 forms a hydrophobic platform close to the His611 imidazole side chain, exposes one face of its indole ring to the solvent, and could therefore stack on a tRNA base. The lack of activity for the Trp679Ala mutant is in agreement with this proposal. Site-directed mutagenesis allowed us to map the colicin D active site and to identify residues critical for cytotoxicity (Lys 608, Lys 610, His611, Arg651, Ser677 and Trp679).

The substrate specificity of the tRNase activity of colicin D is remarkably narrow. This activity is conferred by a small domain carrying a modestly sized active site cleft, and subtle recognition mechanisms must be at work to achieve this specificity. In the four tRNA^{Arg} isoacceptors, position 38 is occupied by an adenine that is not involved in Watson-Crick base pairing. In general, in the 3D structures of free tRNA, the backbone phosphate moiety of nucleotide 38 is solvent exposed and its base makes a hydrogen bond with the base at position 32. In the structure of the complex between arginyl-tRNA synthetase and yeast tRNA^{Arg}, the adenine ring at position 38 flips out of the anticodon loop to interact with the enzyme (Delagoutte *et al*, 2000). It is therefore tempting to speculate that upon binding to colicin D, the adenine base will do the same and stacks on the exposed face of the Trp679 side chain. How this can result in a highly specific cleavage will have to await structural information on colicine D-tRNA^{Arg} complexes.

Interaction between the colicin D catalytic domain and ImmD

The crystal structures of several nuclease domains (colicins E3, E7 and E9) in complex with their bound immunity proteins (Kleanthous *et al*, 1999a; Ko *et al*, 1999; Carr *et al*, 2000), and of the complex between the full-length colicin E3 and its inhibitor Imm3 (Soelaiman *et al*, 2001), have been reported. In combination with protein engineering experiments, these structures have revealed a common mechanism for nuclease inhibition by the respective Imm proteins. The active site remains exposed, but the Imm protein prevents the

access of incoming bulky substrates (ribosome or genomic DNA) to the catalytic groups (Kolade *et al*, 2002). The predominant interactions involved in complexes between the colicin E7 and E9 DNase domains and their Imm proteins were shown to be charged and hydrophobic, respectively (Kuhlmann *et al*, 2000). The core of the E-type DNase active site is formed by a H-N-H motif originally found in intron-encoded homing endonucleases (James *et al*, 2002). Specific ImmE7 binding is conferred by a small number of critical amino-acid residues (Lu *et al*, 1999). Such a mode of inhibition contrasts with classic enzyme inhibitor complexes such as barnase-barstar (Buckle *et al*, 1994), uracil-DNA glycosylase-uracil glycosylase inhibitor protein (Savva and Pearl, 1995), where the inhibitors completely block access to the active sites and interact with well-conserved residues.

The crystal structure of the colicin D catalytic domain in complex with ImmD allows a comparison to other immunity proteins and between the different modes of inhibition used to block colicin activity. Comparison of ImmD with other immunity proteins shows that the DNase colicin inhibitors (ImmE7, ImmE8 and ImmE9) are of identical size (87 amino acids) and have a similar four-bundle architecture (Kleanthous *et al*, 1999b; Ko *et al*, 1999). However, the lengths and the relative orientations of the helices within the bundle are very different and their structure cannot be superposed on that of ImmD. Similarly, the immunity protein of colicin E3, which inhibits an rRNase-type colicin, adopts a totally different α/β fold (Soelaiman *et al*, 2001). In the structure of the colicin D-ImmD complex, the ImmD protein associates by inserting two helices into the active site depression, thereby completely blocking the presumed active site pocket. There is therefore a perfect overlap between the colicin D region recognized by ImmD and the catalytic site defined by mutagenesis experiments (Figure 2D and E).

The full-length colicin D protein was previously found to be susceptible to cleavage in cell extracts, in a process dependent on the leader peptidase LepB (de Zamaroczy *et al*, 2001). The cleavage site has been localized between residues Lys607 and Lys608 (M de Zamaroczy, unpublished data, 2003) and ImmD protects colicin D against this cleavage. The structure we report here explains the protection observed, as these two Lys residues, at the start of the catalytic domain, are completely masked in the colicin D-ImmD complex. Thus, during secretion, ImmD associated with colicin D simultaneously blocks the toxic tRNase activity and the possibility of a proteolytic cleavage.

The present structure is the first example of an Imm protein inhibiting a colicin by completely blocking the active site, rather than by preventing access of the substrate to the active site through binding to an adjacent site. Hence, the mechanism of inhibition of ImmD resembles the more classic enzyme-inhibitor complexes such as barnase-barstar (Buckle *et al*, 1994) or trypsin-BPTI (Huber *et al*, 1974). In the complex, the interacting surface is dominated by charge-complementary interactions. Especially noteworthy is the match between the positively charged clamp on helix 1 from colicin D and a negatively charged patch on ImmD (Figure 2F). Mutation of the residues responsible for the positively charged region on colicin D abolishes or reduces colicin D cytotoxicity. This charged surface could then play an important role for the tRNA substrate binding by neutralizing the backbone phosphate groups. The ImmD protein

presents a negatively charged surface to this region of colicin D, thus mimicking the phosphate groups of tRNA substrate. A similar mechanism was reported for the inhibition of the uracil-DNA glycosylase by its inhibitor protein (Savva and Pearl, 1995).

Several residues in helix $\alpha 4$ were shown to be essential for the inhibition of colicin D. These residues are distant from the protein surface area located between ImmD and the colicin D active site, and most probably contribute to anchor ImmD to a second domain more towards the N-terminus of colicin D. This may maintain an overall structure of the colicin complex better optimized for its immunity during secretion, and/or for receptor recognition during target cell invasion. Consistent with this proposal, the wild-type colicin E3/ImmE3 complex, comprising a second interaction between ImmE3 and the translocation domain, dissociates two orders of magnitude more slowly than the complex simply formed by the catalytic domain and ImmE3 (Walker *et al*, 2003). The bipartite interaction of ImmE3 with colicin E3 (Soelaiman *et al*, 2001) suggested a cascade mechanism for colicin E3 penetration into target cells. The binding of the extended coiled-coil receptor-recognition domain of colicin E3 to BtuB places the N-terminal translocation domain close to a neighbouring OmpF porin (Kurusu *et al*, 2003). According to the proposed mechanism, the subsequent interaction with OmpF (second receptor) would abolish the contact of the translocation domain with the ImmE3, which in turn could weaken the binding of ImmE3 to colicin and thereby trigger the unfolding necessary for translocation of the C-terminal catalytic domain (Zakharov and Cramer, 2002). However, the N-terminal part of colicin B, highly conserved in colicin D, was recently shown (Hilsenbeck *et al*, 2004) to adopt a single-domain structure comprising both the receptor recognition and the translocation regions. This structure is completely different from that of the corresponding regions in the E-type colicins. In addition, the immunity proteins of D- and E-type colicins adopt different folds. Altogether, these observations suggest that the ImmD may interact with the central domain of colicin D, of unknown function, and/or with the translocation domain common to colicin B in a manner completely different from what was observed for colicin E3. Further studies will be required to identify the precise target and the functional role of the interaction implicating helix $\alpha 4$ of ImmD, taking into account the fact that no second receptor, involved in the translocation of colicin D across the outer membrane, has so far been identified.

Materials and methods

Expression, purification and activity assay of colicin D

The purification of the native colicin D in complex with its immunity protein was performed from the supernatant of a culture of *E. coli* strain K12 carrying pColD-CA23 (pJF129) induced with mitomycin C (200 μ g/l) (Frey *et al*, 1986). Labelling with selenomethionine (Se-Met) was achieved by supplementing a culture in minimal medium with L-Se-Met (70 mg/l), and six other L-amino acids (Lys, Phe and Thr at 125 mg/l each; Ile, Leu, Val, each at 62.5 mg/l) to repress the methionine biosynthesis pathway, at an OD₆₀₀ of 0.7 and after induction by mitomycin C (Doublié, 1997). Expression was continued for 4.5 h. The colicin complex was purified by successive chromatographic steps on Q sepharose FF and Mono Q (Pharmacia) columns (de Zamaroczy *et al*, 2001), followed by (NH₄)₂SO₄ precipitation (60%), resuspension of the precipitate and gel filtration on a HiLoad Superdex 200 column. The pooled colicin D/ImmD complex was first concen-

trated on a Mono Q column and finally by ultrafiltration (Amicon Ultra-4 10k NMWL; Millipore). The cytotoxicity of the purified colicin D/ImmD was verified *in vivo*, by a halo (zone of inhibition) assay. Se-Met incorporation into the purified complex was assayed by mass spectrometry.

Spontaneous fragmentation of colicin D–ImmD complex

Purified colicin D–ImmD complex, usually stored in 20 mM phosphate Na buffer (pH 7.0), 0.15 M NaCl, is stable for several months at 4°C, as judged by the absence of proteolytic degradation and a constant killing activity over time *in vivo*. Nevertheless, spontaneous fragmentation was regularly observed during colicin D crystallization trials, after storage for 8–12 weeks at 4–20°C, in 20 mM Tris buffer (pH 8.0). The fragments identified from SDS-PAGE consisted mainly of the colicin D C-terminal 12 kDa peptide and the immunity protein. The fragmentation was further analysed by N-terminal peptide sequencing colicin D peptides. In the presence of ‘complete’ protease inhibitor complex (Roch), the spontaneous fragmentation over similar periods of storage was eliminated. Historically, some nuclease colicin molecules have been observed to undergo auto-fragmentation on storage, for instance, in the cases of colicin E3 and cloacin DF13 complex (Lau and Richards, 1976; de Graaf *et al*, 1978). More recently, the proteolytic auto-fragmentation of botulinum A neurotoxin LC was reported and shown to be stimulated by Zn²⁺ (Ahmed *et al*, 2001).

Crystallization and resolution of the structure

Native and Se-Met-labelled protein samples were stored in 20 mM Tris-HCl (pH 8). Crystals for the native protein were grown at 18°C from a 1:1 μ l mixture of 26 mg/ml protein solution with 30% PEG 400, 0.2 M MgSO₄, in the presence of *N*-octyl β -D-glucoside detergent. Se-Met-labelled protein crystals were grown by mixing 1 μ l of a 20 mg/ml protein solution with 1 μ l of the reservoir solution composed of 24% PEG 8K, 0.1 M Na citrate (pH 6.5). Analysis of the crystal content by mass spectrometry, in agreement with the mentioned biochemical analysis, revealed that it contained the intact immunity protein, but only a fragment of colicin D, corresponding to the catalytic domain. For data collection, the crystals were transferred into a cryoprotectant solution composed of the mother liquor and 30% glycerol. Crystals of the native and Se-Met-labelled protein diffracted to 2.0 and 3.0 Å, respectively, on beamline BM30A at the European Synchrotron Radiation Facility (ESRF, Grenoble, France; Roth *et al*, 2002).

The structure was determined using SAD X-ray diffraction data, collected from the Se-Met derivative crystal at 3 Å resolution. Data were processed using the HKL package (Otwinowski and Minor, 1997). The space group was P4₁2₁2 ($a = b = 61.9$ Å, $c = 150.0$ Å) with one complex per asymmetric unit. Three Se atom sites out of five were found with the program SOLVE in the 80–3.6 Å resolution range (Terwilliger, 1999). After phase extension to 3 Å and solvent flattening with the program RESOLVE (Terwilliger and Berendzen, 1999), the quality of the electron density map allowed the manual construction of most of the secondary structure elements with the ‘TURBO’ molecular modelling program (Roussel and Cambillau, 1991). This partial model was then refined against the 2 Å data set with the Arp/wARP program that allowed automated construction of 98% of the model (Perrakis *et al*, 1999). After completion of the colicin D–ImmD model, the structure was refined with the CNS program (Brünger *et al*, 1998). All the residues are well defined in electron density and fall within the allowed regions of the Ramachandran plot, as defined by the program Procheck (Laskowski *et al*, 1993). Statistics for all the data collections and refinement of the different structures are summarized in Table 1. The atomic coordinates and structure factors for the colicin D–ImmD complex have been deposited into the Brookhaven Protein Data Bank under the accession number 1V74.

Cleavage and killing activities of mutated colicin D molecules

Mutations were introduced both at residues of the 591–697 catalytic fragment of colicin D or at residues of ImmD, by two-step PCR amplification. Employing plasmid pColD as a template, the first PCR used a 5′ mutagenic oligonucleotide overlapping the mutated position, together with a 3′ oligonucleotide complementary to a downstream sequence located close to the *cda* stop codon of the structural gene of colicin D (*cda*) or ImmD (*cdi*). After denaturation, the PCR product was used as a 3′ oligonucleotide in a second PCR amplification, coupled with an upstream 113-mer oligonucleotide,

containing the *tac* promoter, known to be efficient both *in vitro* and *in vivo* (Huang *et al*, 1994), and a 14 nt ribosomal binding site from pColD, to generate whole mutated *cda* and *cda-cdi* molecules. The resulting PCR products were expressed *in vitro* in a coupled transcription–translation, Zubay-S30 system from *E. coli*, optimized for linear DNA templates (Promega) at 37°C for 60 min. tRNA-hydrolysing activity of the mutated colicins D was directly measured from the S30 expression samples, by separation of the intact and cleaved forms of the tRNA^{Arg} on PAGE (10% in the presence of 7 M urea), followed by Northern blot analysis (Tomita *et al*, 2000), using a [³²P]-labelled DNA probe specific for tRNA^{Arg}(CCG). In parallel, the same test was performed with mutated colicin D–ImmD complexes for studying the inhibitory efficiency of the complex. For semiquantitative determination of cytotoxicity and tRNase activity, the amounts of mutated protein in comparison with that of the wild type, were previously determined

from [³⁵S]Met-labelled mutated colicins by phosphorimager (Molecular Dynamics), and found to be similar (data not shown). In parallel, mutated colicin D/ImmD complexes were directly tested for their *in vivo* cytotoxic activity on a lawn of sensitive wild-type C600 strain on LB plates.

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