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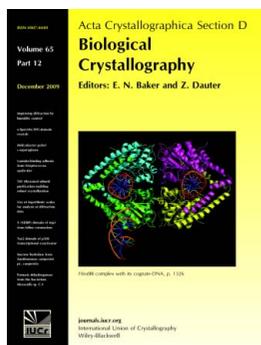
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Structures of restriction endonuclease *HindIII* in complex with its cognate DNA and divalent cations

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The three-dimensional crystal structures of *HindIII* bound to its cognate DNA with and without divalent cations were solved at 2.17 and 2.00 Å resolution, respectively. *HindIII* forms a dimer. The structures showed that *HindIII* belongs to the *EcoRI*-like (α -class) subfamily of type II restriction endonucleases. The cognate DNA-complex structures revealed the specific DNA-recognition mechanism of *HindIII* by which it recognizes the palindromic sequence A/AGCTT. In the Mg²⁺ ion-soaked structure the DNA was cleaved and two ions were bound at each active site, corresponding to the two-metal-ion mechanism.

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PDB References: *HindIII*–cognate DNA complex, 2e52, r2e52sf; metal-ion bound, 3a4k, r3a4ksf.

1. Introduction

Restriction endonucleases are produced in bacteria, archaea and *Chlorella* viruses. These enzymes hydrolyze foreign DNA and thus allow microorganisms to avoid attack by phages *etc.* Based on subunit composition, cofactor requirements, DNA-recognition specificity and mode of action, restriction endonucleases have been classified into four types (Roberts *et al.*, 2003). Type II restriction endonucleases recognize and digest specific palindromic sequences of double-stranded DNA. About 4000 type II restriction endonucleases have been discovered to date. The specificity of the type II restriction endonucleases has made them indispensable tools in biotechnology. However, the tertiary structures of only about 30 type II restriction endonucleases are available at present in REBASE (Roberts *et al.*, 2007). Most of the type II restriction endonucleases have a common sequence motif PD-(D/E)xK (Stahl *et al.*, 1998). Pingoud *et al.* (2005) showed that they contain a core comprised of a five-stranded mixed β -sheet flanked by α -helices and that the second and third β -strands form a scaffold for the catalytic residues of PD-(D/E)xK. Typically, type II restriction enzymes utilize Mg²⁺ ion as a divalent cation. Recent reviews have focused on their structure and function, the role of metal ions and their catalytic mechanisms (Pingoud & Jeltsch, 2001; Pingoud *et al.*, 2005).

The type II restriction endonuclease *HindIII* produced in *Haemophilus influenzae* Rd digests DNA at the palindromic sequence A/AGCTT. *HindIII* is comprised of 300 amino acids and has a molecular weight of 34 950 Da. The amino-acid sequences of this nuclease and the corresponding methyltransferase were deduced from their genes (Nwankwo *et al.*, 1994). We have previously reported protein-engineering studies on *HindIII* (Tang *et al.*, 2000). One mutant, D108L, showed a complete loss of enzyme activity, suggesting that

Table 1
Crystallographic data-collection and refinement statistics.

	Iodine derivative	Native	Mg ²⁺ -soaked
Beamline	BL-5A	NW-12A	BL-6A
Wavelength (Å)	1.5	1.0	1.0
Space group	<i>P2</i> ₁	<i>P2</i> ₁	<i>P2</i> ₁
Unit-cell parameters (Å, °)	<i>a</i> = 83.9, <i>b</i> = 130.9, <i>c</i> = 93.6, β = 110.2	<i>a</i> = 83.1, <i>b</i> = 131.5, <i>c</i> = 93.7, β = 111.2	<i>a</i> = 83.5, <i>b</i> = 132.2, <i>c</i> = 94.1, β = 111.0
Resolution (Å)	2.77 (2.87–2.77)	2.00 (2.07–2.00)	2.17 (2.25–2.17)
Unique reflections	48465	130036	100664
Redundancy	3.6	7.6	1.9
Completeness (%)	95.3 (67.4)	99.9 (99.5)	99.8 (98.0)
<i>R</i> _{merge} (%)	9.2 (37.4)	5.6 (14.3)	5.4 (40.4)
<i>I</i> / σ (<i>I</i>)	7.6 (1.6)	23.2 (12.5)	24.7 (3.5)
Mosaicity (°)	1.00	0.25	0.49
Refinement resolution (Å)		2.00	2.17
<i>R</i> _{work} / <i>R</i> _{free} (5% of reflections)		17.7/21.7	17.5/22.1
No. of waters		968	598
R.m.s.d. bond lengths (Å)		0.015	0.02
R.m.s.d. bond angles (°)		1.50	1.84
Average <i>B</i> factor (Å ²)		23.8	36.1

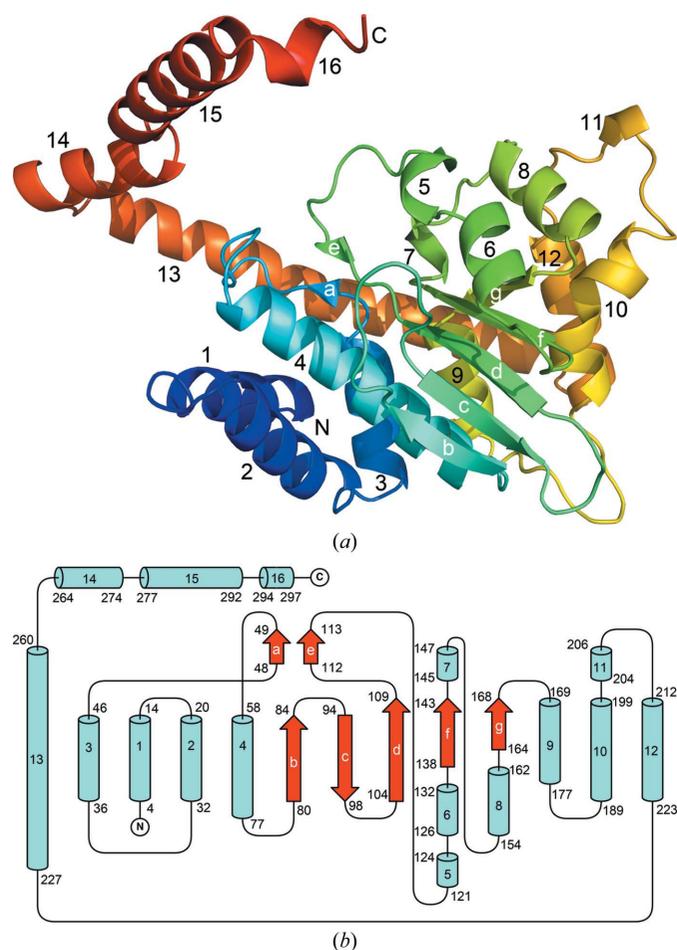


Figure 1
Overall structure of the *Hind*III monomer. (a) *Hind*III monomer with labelled helices and β -strands. (b) Topology diagram of *Hind*III. β -Sheets are shown in red and helices are shown in cyan.

Asp108 is located in the active site of the enzyme. We also found that the mutant E86K showed higher activity than the wild-type enzyme (Tang *et al.*, 1999). However, the tertiary structure of *Hind*III has not been determined. To facilitate

further studies of substrate recognition and enzyme catalysis, we have determined the *Hind*III crystal structure in its cognate DNA complex with and without metal ions.

2. Materials and method

2.1. Protein purification and crystallization

Wild-type *Hind*III was expressed in a His-tagged form (with ten His residues at the N-terminus). The *Hind*III protein was purified by a modification of the method reported previously (Tang *et al.*, 2000). The *Hind*III-RM gene inserted into the pET16b vector was expressed in *Escherichia coli* BL21 (DE3) pLysS.

The bacteria were grown in 3 l LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin, harvested and sonicated to obtain crude extract. The extract in binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 8.0) was applied onto a column packed with His-Bind Resin (Novagen, San Diego, California, USA) in 50 mM NiSO₄. The column was washed with binding buffer and then with wash buffer (60 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 8.0). The protein was eluted by adding 0.05 M EDTA, 0.1 M EDTA, 0.1 M EDTA + 0.1 M imidazole, 0.1 M EDTA + 0.2 M imidazole, 0.1 M EDTA + 0.5 M imidazole and then 1.0 M imidazole to the column in a stepwise manner. *Hind*III protein eluted in the 0.1 M and 0.2 M imidazole fractions. The purified protein thus obtained was dialyzed against a buffer containing 20 mM Tris-HCl pH 8.0 and 0.2 M NaCl overnight. Protease Xa was added to the sample to remove the His tag by treatment at 285 K for 14 h. The sample was then added to the His-Bind resin column and the flowthrough fraction was collected and concentrated to 10 mg ml⁻¹ with Amicon Ultra (Millipore, Billerica, Massachusetts). The weight ratio of protease Xa to *Hind*III was less than 0.1% and therefore no trace was observed in the concentrated sample.

DNA oligonucleotides (5'-GCCAAGCTTGGC-3') were synthesized and purified by HPLC (Hokkaido System Science, Sapporo, Japan). The oligonucleotides for use in crystallization trials were dissolved in Milli-Q reverse-osmose purified water. The oligonucleotide solution was heated to 368 K for 10 min, followed by slow cooling to room temperature. Protein-DNA complexes were prepared by mixing annealed oligonucleotides with *Hind*III and incubating at 277 K overnight. The final protein-DNA complex solution contained *Hind*III protein at a concentration of 6.0 mg ml⁻¹; the DNA duplex was present in a 1.5-fold molar excess relative to the protein dimer.

Crystals of the *Hind*III-DNA complex were grown by the hanging-drop vapour-diffusion method over a reservoir of 12–16% (v/v) PEG 3350, 80–120 mM ammonium acetate. Drops were formed by mixing 1 μl *Hind*III-DNA complex solution

and 1 μ l reservoir solution and were equilibrated over the reservoir at 293 K.

To elucidate the metal-binding structure of the *Hind*III–DNA complex, Mg^{2+} was introduced into the crystals of the *Hind*III–DNA complex using microdialysis buttons. The buttons were left in dialysis solution containing 20 mM $MgCl_2$, 20% glycerol and reservoir solution for one week.

2.2. Data collection

The initial phases were determined at 2.8 Å resolution by the SAD method using an iodinated derivative. Tyrosine residues in the *Hind*III–DNA complex crystals were iodinated using the vaporizing-iodine labelling method (Miyatake *et al.*, 2006). The iodine SAD data were collected at 1.5 Å wavelength and all other data sets were collected at 1.0 Å wavelength using flash-frozen crystals at 95 K. Native and iodinated crystals were cryoprotected with 20% (v/v) glycerol prior to cooling. All data sets were collected at the Photon Factory,

Tsukuba, Japan. Native, iodine-derivative and Mg^{2+} data sets were collected on beamlines NW12A, BL5A and BL6A, respectively. Diffraction data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

2.3. Structure solution and refinement

Iodine positions were determined using *SHELXC* and *SHELXD* (Sheldrick, 2008) using the *HKL2MAP* interface (Pape & Schneider, 2004); the iodine sites were refined and the initial SAD phases were calculated using the program *SOLVE* (Terwilliger & Berendzen, 1999). Further phase improvement and auto model building were carried out using *RESOLVE* (Terwilliger, 2003a,b) and *ARP/wARP* (Perrakis *et al.*, 1999). The structure of the *Hind*III–DNA complex was then refined with *REFMAC5* (Murshudov *et al.*, 1997) using 2.0 Å resolution native data and manually fitted with *Coot*

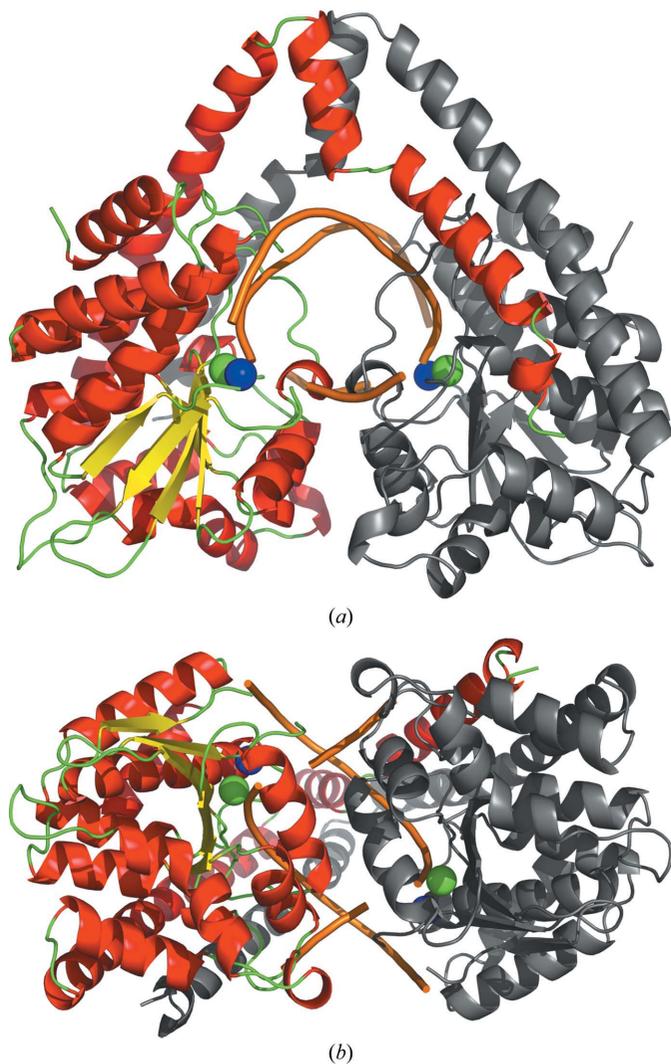


Figure 2
The dimer structure of the *Hind*III–DNA complex. (a) Top view of the complex. (b) View of the complex along the noncrystallographic dyad axis. The two metals observed in the Mg^{2+} -soaked structure are also shown, with Mg^{2+} as blue spheres and Mn^{2+} as green spheres.

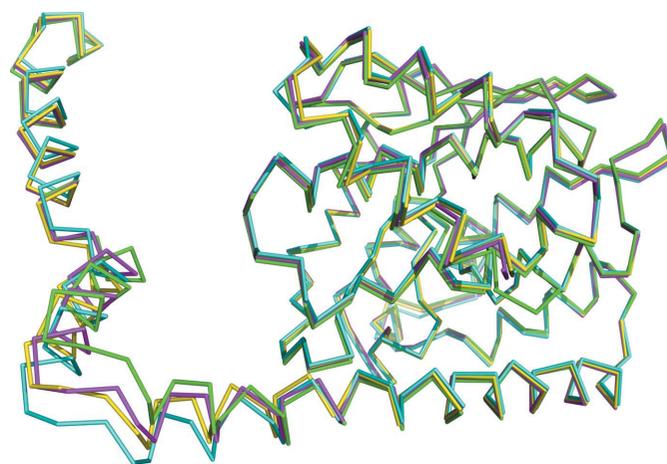


Figure 3
Superposition of the $C\alpha$ ribbons of the four monomers in the asymmetric unit. The monomers are coloured green (A), cyan (B), magenta (C) and yellow (D).

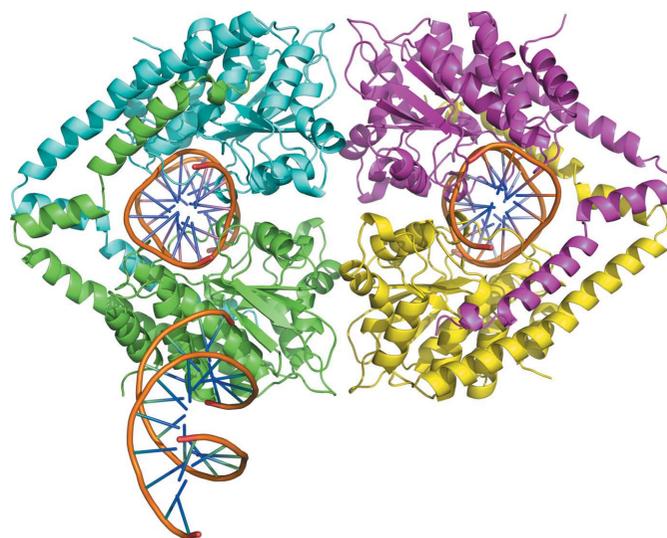


Figure 4
*Hind*III–DNA complex structure observed in the asymmetric unit. The four monomers are coloured green (A), cyan (B), magenta (C) and yellow (D). There is an extra free DNA duplex between monomer A and monomer B in the adjacent asymmetric unit.

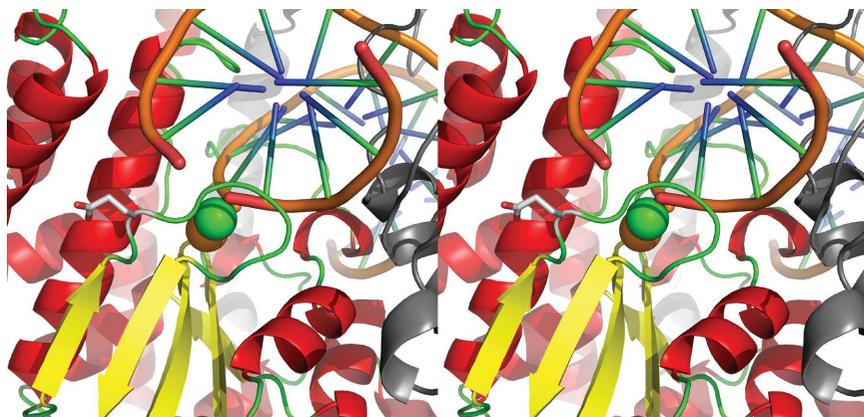


Figure 5
Stereo figure of the flexible-loop region of monomer *D* of the metal-soaked structure. Grey helices are those of monomer *C*. The two metals Mg^{2+} and Mn^{2+} are shown as green and orange spheres, respectively. The side chain of Glu86 is shown as a stick model.

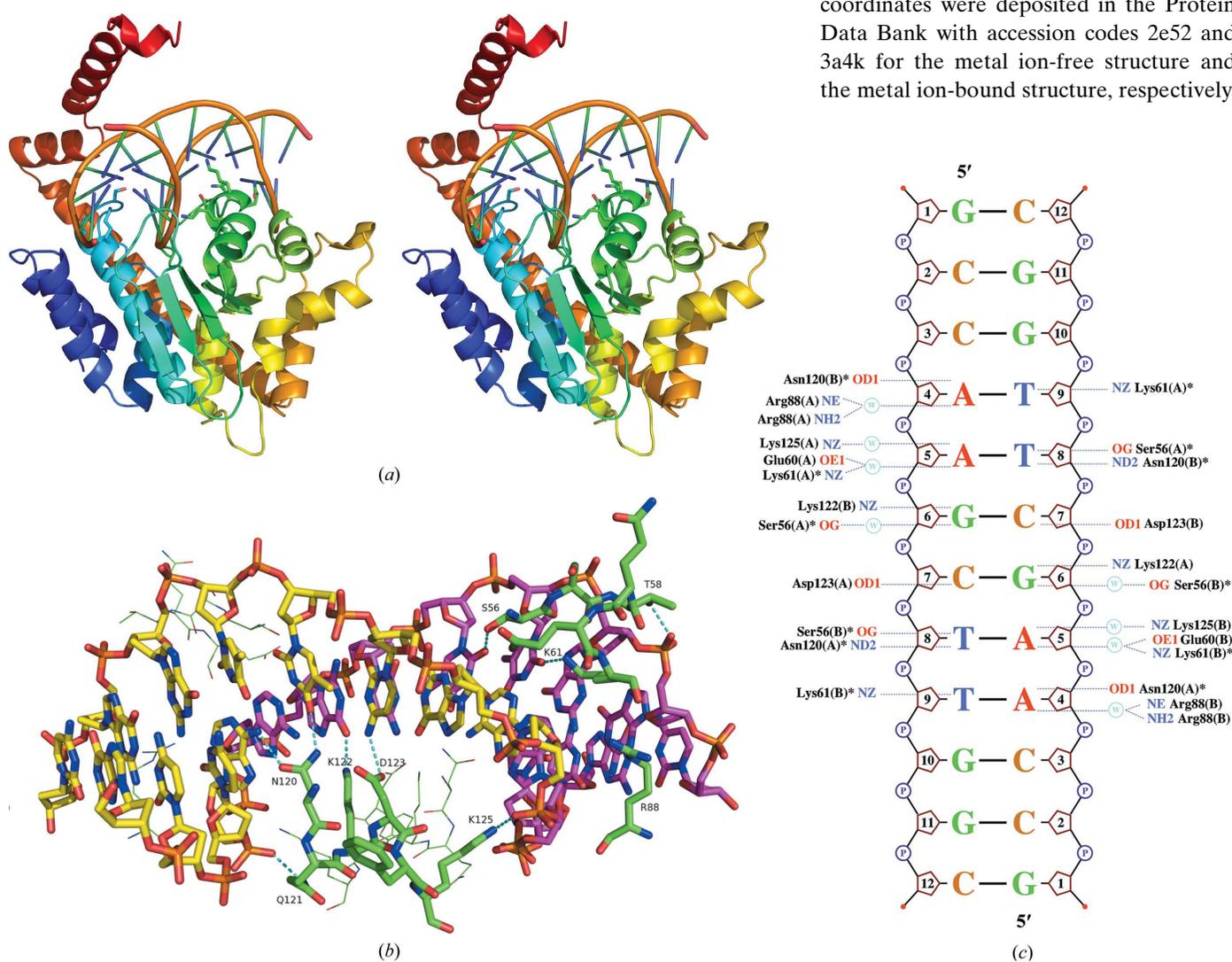


Figure 6
HindIII–DNA interactions. (a) Stereoview of the *HindIII*–DNA interaction. Only one monomer of *HindIII* is shown. Side chains with direct interactions to the bases are shown as stick models. (b) View of the base-pair recognition of *HindIII*, with hydrogen bonds indicated as dashed lines. The DNA is shown in yellow and magenta to indicate the two parts of the post-reaction product. (c) *NUCPLOT* sketch illustrating contacts between *HindIII* and the DNA bases. Contacts between *HindIII* AB dimer and the bases of the DNA strands *E* and *G* are shown. Asterisks indicate residues that appear more than once.

3. Results and discussion

3.1. Overall structure

The *Hind*III monomer (35 kDa) consists of 16 helices and two β -sheets: one small sheet consisting of two short strands and a larger sheet comprised of five β -strands (Fig. 1). Helices 7, 11 and 16 are 3_{10} -helices. Helix 10 is a proline-kinked helix with Pro195 located near its centre. A β -sheet flanked by α -helices is a core structure of restriction endonucleases (Venclovas *et al.*, 1994). *Hind*III also has this core structure ($\alpha 4$, βb , βc , βd , $\alpha 6$, βf , $\alpha 8$, βg , $\alpha 9$). The three key residues of the canonical PD-(D/E)xK catalytic motif, Asp108, Ala109 and Lys110, are located in this larger β -sheet. As βg is parallel to βb , *Hind*III belongs to the *Eco*RI-like (α -class) subfamily (Venclovas *et al.*, 1994; Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). Based on the classification of Niv and coworkers, *Hind*III belongs to class I, H1-S-H2-up, and H2 ($\alpha 9$ in *Hind*III) is almost perpendicular to the β -strands (Niv *et al.*, 2007).

The *Hind*III homodimer measures approximately $70 \times 80 \times 50 \text{ \AA}$ (Fig. 2). The dimer formation of *Hind*III is interesting. The long arm consisting of the C-terminal four helices ($\alpha 13$, $\alpha 14$, $\alpha 15$ and $\alpha 16$) protrudes from the core and interacts with its counterpart in the dimer. In particular, the long helix $\alpha 13$ and three helices ($\alpha 14$, $\alpha 15$ and $\alpha 16$) of the adjacent monomer mainly make contact *via* hydrophobic interactions and form a hinge-like structure. Flexibility of the hinge can be observed when the structures of the four monomers in the asymmetric unit are superposed. The loop between $\alpha 13$ and $\alpha 14$ shows a displacement of more than 6.5 \AA (Fig. 3).

In the crystal, there are four protein monomers and three DNA duplexes in an asymmetric unit. The DNA strands are uncleaved in the cocrystal structure without metals. Protein subunits *A* and *B* or *C* and *D* form dimers which encircle double-stranded DNA molecules. In the asymmetric unit, the *Hind*III dimers form a tetramer with 222 symmetry (Fig. 4). Apart from the tetramer of the asymmetric unit, *PISA* (Krissinel & Henrick, 2007) indicated that only the dimers are potentially biologically relevant, which is also consistent with the estimated molecular weight of the DNA complex on gel-filtration chromatography (data not shown). Interestingly, an extra DNA duplex, the occupancy of which was fixed to 0.7 in the final refinement cycles, exists like a 'shore' between the artificial tetramers. This extra DNA duplex may play an important supporting role in the crystal packing of this *Hind*III–DNA complex.

In the Mg^{2+} -soaked structure eight metal peaks were found in the asymmetric unit, indicating two ions per active site. The DNA strands were cleaved in the crystal. No large structural changes were observed between the structures with and without cations. The overall structure of the *Hind*III–DNA complex was retained, but electron density in a loop between Leu85 and Gly89 was poor in monomers *A*, *B* and *C*. Moreover, the Arg88 residues of these three monomers were completely disordered and their structures could not be built in the model. However, the electron density was clear for the *D* chain and a model of the loop could be constructed with

side chains. The loop conformation of the *D* chain was almost the same as in the metal-free structure. As shown in Fig. 5, this loop is located near the active site, which it covers. Mutational analyses of *Hind*III showed that the E86K mutant had higher activity (Tang *et al.*, 2000). The k_{cat} of the E86K mutant was twice that of the wild type. As shown in the figure, Glu86 is located in the loop. The flexibility and charge distribution of this loop may be important in the transfer of metal ions and the mutation may affect the turnover of *Hind*III.

3.2. Specific DNA recognition

*Hind*III recognizes a specific 6 bp sequence with completely twofold symmetry. Contacts between *Hind*III and the bound DNA are shown in Figs. 6(a) and 6(b) and schematically in

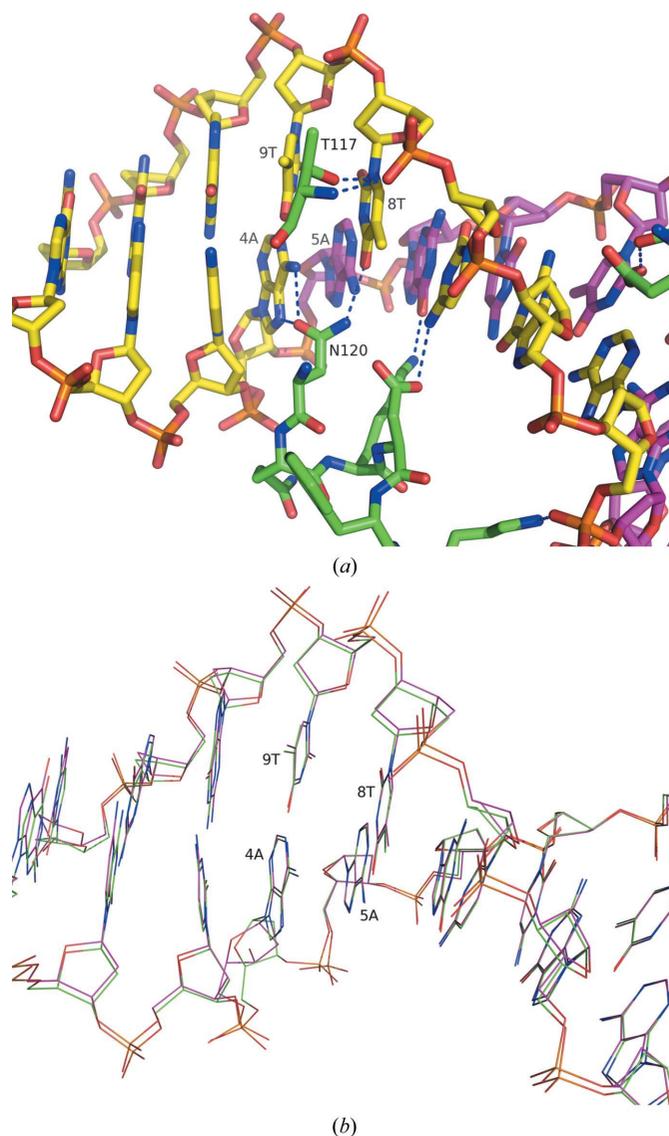


Figure 7
DNA structure near the scissile bond. (a) View of the *Hind*III–DNA interactions, with hydrogen bonds indicated as dotted lines. Residues of *Hind*III are coloured green and the DNA is shown in yellow and magenta to indicate the two parts of the post-reaction product. (b) Superposition of the DNA structures before and after the scission reaction. DNA before the reaction is shown in green and the cleaved DNA is shown in magenta.

Fig. 6(c). The four residues that act at the major groove, Asp120, Lys122, Asp123 and Lys125, are on the short helix $\alpha 5$. Of the residues that act at the minor groove, Glu60 and Lys61 are located at the N-terminal end of helix $\alpha 4$, Ser56 is located in a loop between helices $\alpha 3$ and $\alpha 4$ and Arg88 is in the loop between the βb and βc strands. In the major groove, six direct

hydrogen bonds and several water-mediated hydrogen bonds are formed between the protein and the bases of the specific sequence A/AGCTT of the DNA. Asn120 O ^{$\delta 1$} forms a hydrogen bond to N6 of adenine 4A. Asn120 N ^{δ} also makes direct contact with O4 of thymine 8T. Lys122 N ^{ζ} makes direct contact with O6 of guanine 6G and Asp123 O ^{$\delta 1$} makes direct contact with N4 of cytosine 7C. Lys125 N ^{ζ} forms a water-mediated hydrogen bond to N7 of adenine 5A. In the minor groove, two direct hydrogen bonds and three water-mediated hydrogen bonds are formed to the specific bases. Lys61 N ^{ζ} makes direct contact with N2 of thymine 9T. Ser56 O ^{γ} makes contact with O2 of thymine 8T. Contacts between Arg88 N ^{$\eta 2$} and N ^{ϵ} and N3 of adenine 4A, between Lys61 N ^{ζ} and Glu60 O ^{$\epsilon 1$} and N3 of adenine 5A and between Ser56 O ^{γ} and N3 of guanine 6G are water-mediated. Direct interactions with the bases by *Hind*III are only formed to part of the specific sequence, with most occurring in the major groove. No direct contacts were identified using *NUCPLLOT* (Luscombe *et al.*, 1997) to the bases of the nonspecific sequence at both ends of recognition sequence, although there are many interactions between the sugar-phosphate backbone and the protein in this region, as observed for other endonucleases (Martin *et al.*, 1999).

The conformation of the bound DNA is distorted from its ideal B-DNA structure. The overall amount of DNA distortion was about 57° and that of shortening was about 8%, while the DNA distortion and shortening of the extra free DNA found as a 'shore' between the artificial tetramers were 12° and 0.6%, respectively, as determined using the program *CURVES* (Lavery & Sklenar, 1988). *Hind*III–DNA interactions and superposition of DNA structures around the scissile bond before and after the reaction are shown in Fig. 7. The overall DNA structures are almost the same, but the position of the ribose of adenosine 4A is significantly shifted when the phosphate backbone is broken, even though the purine position does not change. As shown in the figure, interactions between Asn120 and adenine 4A cause the purine to tilt about 25° from the pyrimidine of its counterpart thymine 9T, resulting in an accumulation of strain in the DNA structure. In addition, Thr117, which forms hydrogen bonds to the backbone phosphate of thymine 8T, is the only residue in the disallowed region of the Ramachandran plot.

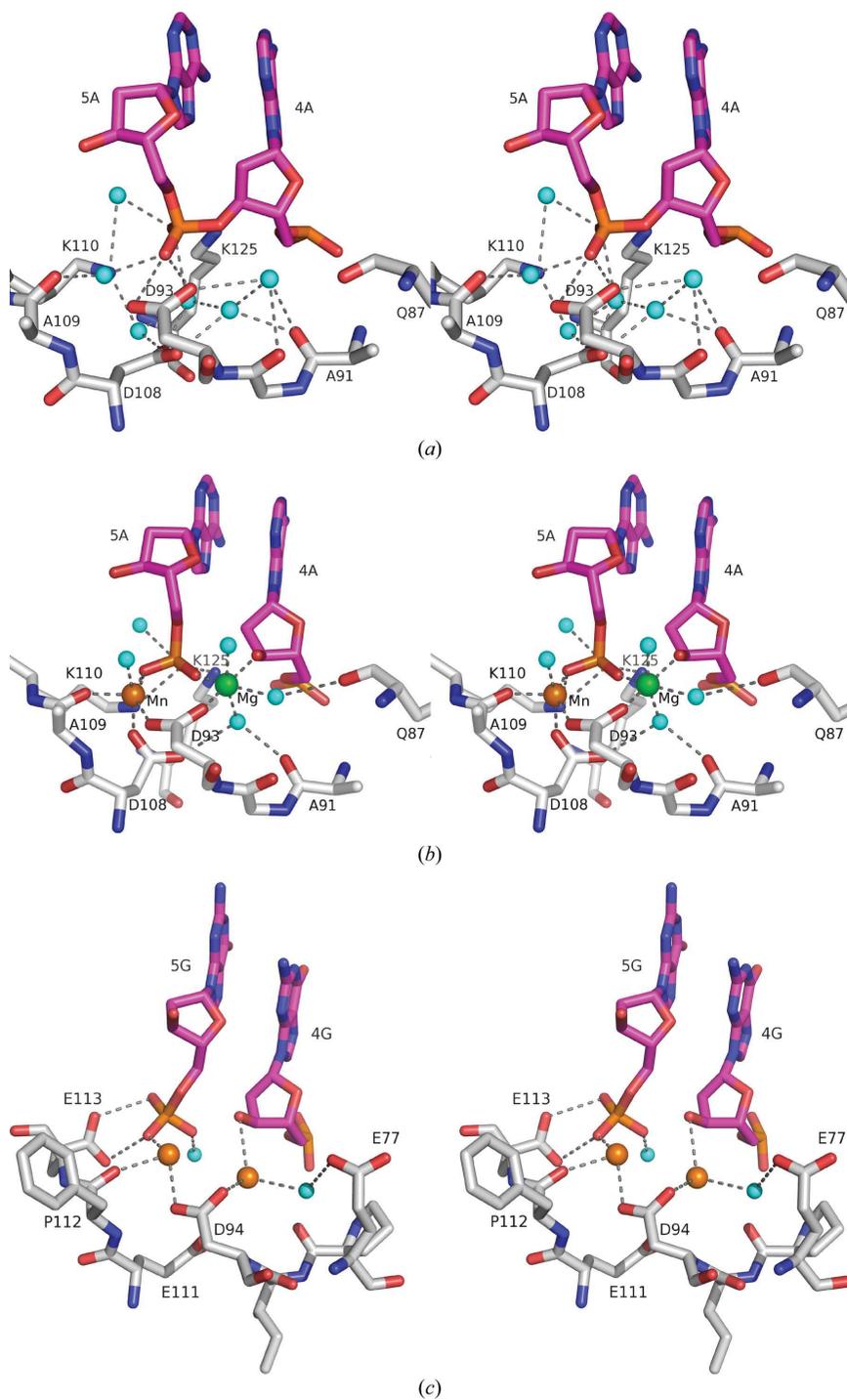


Figure 8

Active-site arrangement. (a) Stereoviews of the active site of the metal-free *Hind*III–DNA complex, (b) of that of the Mg²⁺-soaked structure and (c) of the active-site structure of *Bam*HI. Water molecules are depicted as blue spheres and Mg²⁺ and Mn²⁺ are shown as green and orange spheres, respectively, with hydrogen bonds shown as dashed lines.

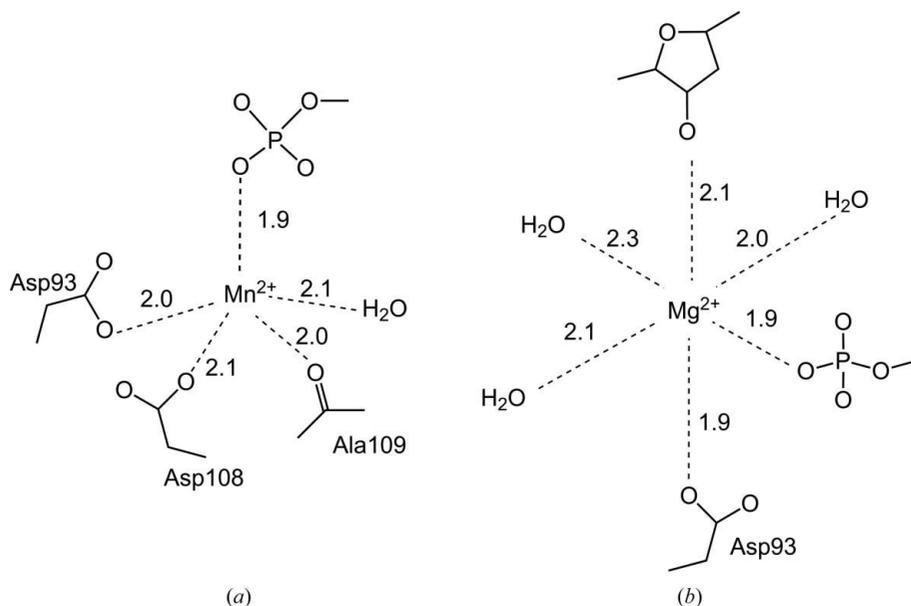


Figure 9
Diagrammatic representations of the metal ion-coordination geometries. Mn^{2+} A site (a) and Mg^{2+} B site (b) are shown with the distances of each hydrogen bond.

Table 2

Refined temperature factors of the metal ions.

Refined B factors of the A site are shown using Mn^{2+} and Mg^{2+} as the metal ions. Those of the B-site Mg^{2+} ions are also shown for comparison.

A site	Refined B factor (\AA^2)		B site	Refined B factor (\AA^2)
	Using Mn^{2+}	Using Mg^{2+}		
<i>O</i>	27.05	7.34	<i>S</i>	30.86
<i>P</i>	32.61	12.28	<i>T</i>	34.80
<i>Q</i>	31.64	11.49	<i>U</i>	37.11
<i>R</i>	33.98	13.34	<i>V</i>	31.85

3.3. Active site

In the Mg^{2+} -soaked crystal two metal ions were found in the active site of each chain and the DNA was cleaved (Fig. 8). The presence of the two metal ions in the active centre of the Mg^{2+} -soaked structure near the cleaved phosphate allowed us to unambiguously determine the catalytic mechanism of *HindIII* as the two-metal-ion mechanism (Beese *et al.*, 1993; Viadiu & Aggarwal, 1998). The distance between the two metal ions is 3.98 Å and is consistent with the transition-state model of the two-metal-ion mechanism. Interestingly, the two metals seem to be different although the crystal was soaked with Mg^{2+} . The metal ions located at the B site seemed to be Mg^{2+} , but those at the A site were likely to be Mn^{2+} . As we could not reproduce this high-resolution DNA-complex crystal, we were not able to confirm this using the anomalous signal of Mn^{2+} with longer wavelength X-rays, but the temperature factors of the refined metal sites suggested that the sites probably contained Mn^{2+} and not Mg^{2+} (Table 2). The temperature factor of the A-site metal tends to be lower than that of B site. For example, in the case of *NgoMIV* the B factor of the A-site Mg^{2+} is 11.0–15.0 Å², while that of the B site is 22.0–31.0 Å² (Deibert *et al.*, 2000). For the *HindIII* structure, however, the temperature factors of the A-site metal ions are

not consistent with those of the neighbouring atoms when using Mg^{2+} as the metal ion. As MgCl_2 was used in the soaking experiment, the Mn^{2+} ion at the A site is probably the preferred selection by *HindIII* from the trace elements present. It is not clear why Mn^{2+} is preferred over Mg^{2+} , but the difference in reaction kinetics between Mn^{2+} and Mg^{2+} in *EcoRV* has been discussed based on the intrinsic affinities of these ions for phosphates (Sam & Perona, 1999) and seems to be consistent with the observation that the Mn^{2+} concentration optimum is about ten times lower than that of Mg^{2+} for many PD-(D/E)_xK-family endonucleases (Pingoud *et al.*, 2009).

The geometry around the two metal ions is shown in Fig. 9. The A-site Mn^{2+} ion is pentacoordinated and the B-site Mg^{2+} ion is hexacoordinated. The coordination architecture of the two ions is as follows. Asp93 O^{δ1}, 5'-O3P and 3'-O3 of cleaved DNA and three water molecules ligate the B-site Mg^{2+} ion. Asp93 O^{δ2}, Asp108 O^{δ2}, the main-chain O atom of Ala109, 5'-O1P of cleaved DNA and one water molecule bind to the Mn^{2+} ion at the A site. The coordination distances are reasonable compared with the distributions in the *MESPEUS* database (Hsin *et al.*, 2008). Asp93 of *HindIII* corresponds to Asp94 of *BamHI*, which is important for two-metal binding, as shown in Fig. 8. Our previous studies showed that another aspartic acid, Asp108, is also important for *HindIII* activity (Tang *et al.*, 1999, 2000). As can be seen in Figs. 8(a) and 8(b), the conformation of Asp108 is changed to coordinate to the two metal ions and Asp108 interacts directly with Mn^{2+} at the A site and with Mg^{2+} at the B site by water-mediated hydrogen bonding.

In summary, we have presented the three-dimensional crystal structure of *HindIII* bound to cognate DNA in metal-free and Mg^{2+} -soaked forms. The DNA was cleaved in the Mg^{2+} -soaked form, indicating that the solved structure is the active form. Further studies are required as the metal ion at the A site is responsible for activating the nucleophile. However, other crystallization conditions are required that do not lead to the free extra DNA 'shore' between the artificial tetramers, as the present crystallization conditions were poorly reproducible.

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