Mapping metal ions at the catalytic centres of two intron-encoded endonucleases

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Divalent metal ions play a crucial role in forming the catalytic centres of DNA endonucleases. Substitution of Mg^{2+} ions by Fe^{2+} ions in two archaeal intron-encoded homing endonucleases, *I-DmoI* and *I-PorI*, yielded functional enzymes and enabled the generation of reactive hydroxyl radicals within the metal ion binding sites. Specific hydroxyl radical-induced cleavage was observed within, and immediately after, two conserved LAGLIDADG motifs in both proteins and at sites at, and near, the scissile phosphates of the corresponding DNA substrates. Titration of Fe^{2+} -containing protein–DNA complexes with Ca^{2+} ions, which are unable to support endonucleolytic activity, was performed to distinguish between the individual metal ions in the complex. Mutations of single amino acids in this region impaired catalytic activity and caused the preferential loss of a subset of hydroxyl radical cleavages in both the protein and the DNA substrate, suggesting an active role in metal ion coordination for these amino acids. The data indicate that the endonucleases cleave their DNA substrates as monomeric enzymes, and contain a minimum of four divalent metal ions located at or near the catalytic centres of each endonuclease. The metal ions involved in cleaving the coding and the non-coding strand are positioned immediately after the N- and C-terminally located LAGLIDADG motifs, respectively. The dual protein/nucleic acid footprinting approach described here is generally applicable to other protein–nucleic acid complexes when the natural metal ion can be replaced by Fe^{2+} .

Keywords: archaeal introns/homing endonucleases/hydroxyl radicals/metal ions/protein footprinting

Introduction

Insight into the structure of the catalytic site of deoxyribonucleases is mainly based on crystallographic studies of, for example, DNase I, 3'-5' exonuclease of DNA polymerase I, nuclease P1 and the restriction endonucleases *EcoRV* and *EcoRI*, which show that divalent metal ions are coordinated to the cleavage site phosphates (Suck and Oefner, 1986; Beese and Steitz, 1991; Volbeda *et al.*, 1991; Vipond *et al.*, 1995). In the present study, we

examine for the first time the coordination and functional significance of divalent metal ions in another class of endonucleases, the intron-encoded homing enzymes. These enzymes are encoded by group I introns, archaeal introns or are expressed as inteins (reviewed in Lambowitz and Belfort, 1993), and they specifically cleave their intron⁺/intein⁺ DNA alleles near the site of intron/intein insertion, as a first step in an intron/intein homing reaction, that was first described for the omega intron of *Saccharomyces cerevisiae* mitochondrial 23S-like rDNA (Jacquier and Dujon, 1985). Many homing enzymes—including all inteins and those encoded by archaeal introns—contain two copies of an, often highly degenerate, LAGLIDADG motif. Four LAGLIDADG-type proteins encoded by hyperthermophilic archaeal introns are known (Kjems and Garrett, 1985; Dalggaard and Garrett, 1992; Burggraf *et al.*, 1993). Of these, the proteins encoded by the intron of *Desulfurococcus mobilis* 23S-like rRNA, *I-DmoI* and intron 1 of *Pyrobaculum organotrophum* 23S-like rRNA, *I-PorI*, are known to cleave DNA from intron⁺ alleles site-specifically *in vitro* (Dalggaard *et al.*, 1993; Lykke-Andersen *et al.*, 1994). Moreover, the *D. mobilis* intron can move intercellularly and home in an intron⁺ population of *Sulfolobus acidocaldarius* (Aagaard *et al.*, 1995).

LAGLIDADG-type homing enzymes are highly specific for their target sequences, recognizing up to 20 base pairs, although some sequence redundancy is allowed (Colleaux *et al.*, 1988; Sargueil *et al.*, 1990; Marshall and Lemieux, 1992; Wernette *et al.*, 1992; Schapira *et al.*, 1993; Lykke-Andersen *et al.*, 1994; Aagaard *et al.*, 1997). With one exception, the recognition sequences are non-palindromic and experimental evidence suggests that the endonucleases bind as monomers to their DNA substrates, primarily in the major groove (Lykke-Andersen *et al.*, 1996; Aagaard *et al.*, 1997). DNA cleavage requires divalent cations and generates four nucleotide 3'-staggered cuts with 5'-phosphates. Insight into the functional domain structure of the homing endonucleases is limited. A biochemical study, based on protein footprinting, identified four regions which may be involved in DNA recognition (Lykke-Andersen *et al.*, 1996). Furthermore, studies with enzyme mutants have indicated that the conserved LAGLIDADG motifs are important for catalysis (Gimble and Stephens, 1995; Henke *et al.*, 1995), and it has been speculated that they may be involved in the metal ion coordination (Gimble and Stephens, 1995; Turmel *et al.*, 1995).

In order to address this question, we employed an Fe^{2+} -hydroxyl radical approach, previously used to map metal binding sites on the *Escherichia coli* glutamine synthetase (Farber and Levine, 1986), the Tet repressor (Ettner *et al.*, 1995), pigeon liver malic enzyme (Wei *et al.*, 1995) and the *E. coli* RNA polymerase (Zaychikov *et al.*, 1996). The divalent metal ion binding sites on the two homing endonucleases, *I-DmoI* and *I-PorI*, were mapped in the

absence and presence of their DNA substrates, using a dual Fe^{2+} -hydroxyl radical approach for both ^{32}P -end-labelled proteins and DNA. The results were combined with those of competition experiments, using Ca^{2+} ions as competitor, and analyses of endonucleases mutated at potential metal ion coordinating amino acids, to yield insight into metal ion coordination within the catalytic sites of these enzymes.

Results

Divalent cation requirement for endonucleolytic activity

The archaeal homing enzymes, I-*DmoI* and I-*PorI*, were expressed in *E. coli* from vectors pET-HTG-*DmoI* and pET-HTG-*PorI* (Lykke-Andersen *et al.*, 1996). They contain a glutathione S-transferase (GST) tag at the C-terminus, and were purified in one step on glutathione-Sepharose, before removing the GST tag by cleavage with thrombin endoprotease. Each protein can also be ^{32}P -end-labelled at a heart muscle kinase site, positioned at the N-terminus, using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and heart muscle kinase. After removal of the GST tag, the endonucleases contain a 5- to 10-amino acid extension at each end that does not impair cleavage activity *in vitro* (Lykke-Andersen *et al.*, 1996).

The divalent cation requirements of I-*DmoI* and I-*PorI*, for cleavage of their respective DNA target sites, were investigated (Figure 1). Enzyme-substrate complexes were incubated in the presence of 1 mM of different divalent cations at the optimal cleavage temperatures of 65°C and 80°C for I-*DmoI* and I-*PorI*, respectively (Dalgaard *et al.*, 1993; Lykke-Andersen *et al.*, 1994). I-*DmoI* and I-*PorI* were found to exhibit similar divalent cation dependence, following the relative order of descending reactivity at 1 mM: Mn^{2+} , $\text{Fe}^{2+} > \text{Mg}^{2+} \geq \text{Co}^{2+}$, $\text{Ni}^{2+} > \text{Zn}^{2+}$. Addition of Cu^{2+} , Ca^{2+} and Ba^{2+} produced no activity. Initial cleavage rate experiments were also performed for both endonucleases at Mg^{2+} and Ca^{2+} concentrations from 1 to 20 mM, and while Ca^{2+} did not support cleavage at any concentration tested, raising the Mg^{2+} concentration from 1 to 20 mM increased the cleavage rate ~15-fold (data not shown). Attempts to determine optimal Fe^{2+} concentrations failed due to degradation and aggregation of DNA and protein at high Fe^{2+} concentrations and at long incubation times (data not shown). Importantly, however, for this study, both endonucleases were highly active in the presence of Fe^{2+} ions at 1 mM.

Localizing metal binding sites in the endonucleases

In the presence of hydrogen peroxide (H_2O_2) and ascorbate, Fe^{2+} ions generate hydroxyl radicals that cleave the backbones of polypeptides and polynucleotides. The capacity of Fe^{2+} to function as a metal ion cofactor in the cleavage reactions enabled us to induce reactive hydroxyl radicals at the divalent cation-binding sites of I-*DmoI* and I-*PorI*. By mapping the resulting cleavage of the polypeptide chain and the DNA substrate, it was possible to localize the metal ions in the protein-DNA complex. Hydroxyl radical cleavage sites in the protein moieties were mapped using a recently developed protein foot-printing approach that involves site-specific ^{32}P labelling

Fig. 1. Divalent cation preferences for (A) I-*DmoI* and (B) I-*PorI* cleavage. The DNA substrates for I-*DmoI* (567 bp) and I-*PorI* (527 bp) were 5' end-labelled with ^{32}P , and incubated in the presence of 1 mM of different divalent cations. After incubation, the products were electrophoresed on 5% polyacrylamide-7 M urea gels, and subjected to autoradiography. The specific cleavage products are indicated, and contain 409 and 158 bp fragments for I-*DmoI* and 268 and 259 bp fragments for I-*PorI*.

at either the N- or C-terminus of the proteins (Jensen *et al.*, 1995a). A protein sequence ladder, run alongside the hydroxyl radical cleavage reactions, was generated by partial cleavage of the end-labelled proteins with sequence-specific proteinases, allowing rapid identification of the positions of the metal ion cleavage sites, with an accuracy of about two amino acids. N-terminally labelled I-*DmoI* and I-*PorI* were incubated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2/\text{ascorbate}$, in the absence or presence of their respective DNA substrates, and degradation products were separated on high-resolution SDS-polyacrylamide gels (Figure 2A and C). As expected, the presence of free radicals in the solution gave rise to a weak background of unspecific cleavages for large parts of the proteins (Figure 2A and C, lane 2). Four major hydroxyl radical cleavage sites were always observed for both I-*DmoI* and I-*PorI* and, in contrast to the other cleavages, these sites were all strongly enhanced in the presence of the DNA substrate (Figure 2A and C, compare lanes 2 and 3). Experiments using C-terminally ^{32}P -labelled I-*DmoI* (Figure 2B) and I-*PorI* (data not

Fig. 2. Fe²⁺-induced hydroxyl radical cleavage of I-DmoI labelled at the (A) N-terminus and (B) C-terminus and (C) cleavage of I-PorI labelled at the N-terminus. N-terminally or C-terminally ³²P-labelled endonucleases were incubated without (lane 1) or with Fe²⁺ and H₂O₂ in the absence (lane 2) or presence (lane 3) of DNA substrate, or in the presence of DNA substrate, Fe²⁺, H₂O₂ and EDTA (lane 4). The products were resolved on SDS-polyacrylamide gels and autoradiographed. Sites of Fe²⁺-induced hydroxyl radical cleavage in the presence of DNA substrate are shown by arrows, and the approximate amino acid positions of the cleavages in the endonucleases are indicated, in one-letter code, in brackets. Approximate positions of the LAGLIDADG motifs are denoted by vertical lines on the right of the autoradiograms. The endonucleases were cut with site-specific endoproteases to provide sequence markers (R, K, D, E, Ch); the endoproteases and their specificities are: R, Arg-C (arginines); K, Lys-C (lysines); D, Asp-N (aspartates); E, Glu-C (glutamates > aspartates); Ch, chymotrypsin (primarily aromatic residues). Amino acid assignments, from the endoprotease cleavages, are given in one-letter code to the left in each panel. (D) Primary structure of I-DmoI and I-PorI. The positions of Fe²⁺-induced hydroxyl radical cuts are indicated by arrows and the LAGLIDADG motifs are boxed. The conserved acidic residues which are changed in the mutational analysis (see Figure 5) are indicated below the sequence. Below, a schematic representation of the conserved domains in archaeal homing endonucleases is given, as defined in previous work (Lykke-Andersen *et al.*, 1996). The LAGLIDADG motifs are depicted as cross-hatched boxes and the putative DNA binding domains are drawn as open boxes. The two tandemly repeated domains are indicated, and the Fe²⁺/H₂O₂-induced cleavages, indicated by arrows, are located at similar positions in each domain of the endonuclease.

shown) yielded the same hydroxyl radical cleavage pattern as N-terminally labelled endonucleases, confirming that the four major hydroxyl radical cleavages are primary cleavage events, independent of cleavage elsewhere in the protein. From the migration of the cleavage products relative to the sequence marker bands, they were localized at or near L14, V39, L113 and K130 in I-DmoI and at or near K8, V28, L86 and V109 in I-PorI (Figure 2). Cleavage at the site closest to the C-terminus of I-DmoI (K130) invariably produced a smeared band using N-terminally labelled I-DmoI (Figure 2A, lane 3) and, therefore, the location of this site was determined using C-terminally labelled I-DmoI which produced a sharp band (Figure 2B). The cleavages induced by hydroxyl

radicals generally produced doublet bands for both endonucleases, probably due to the formation of products with plus or minus one amino acid (Platis *et al.*, 1993). The hydroxyl radical cleavages could be inhibited by adding EDTA to chelate the Fe²⁺ ions (Figure 2A–C, lane 4).

Locating metal sites on the DNA substrates

The observation that the presence of the DNA substrate specifically enhanced the four main hydroxyl radical cleavages for each endonuclease, suggests that the DNA actively contributes to the coordination of the metal ions in the protein. To map the metal ion sites on the DNA substrates, 25 bp DNA fragments containing the cleavage sequences for I-DmoI and I-PorI, were 5' end-labelled on

Fig. 3. Fe^{2+} -induced hydroxyl radical cleavage of (A) *I-DmoI* and (B) *I-PorI* DNA substrates. The corresponding 25-mer DNA substrates, containing a 5' end-label at the coding or non-coding strand, were incubated in the presence of Mg^{2+} , Fe^{2+} or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$. Control samples, containing no divalent cation (–), or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ but no protein, are also included. The products were denatured, electrophoresed on 20% polyacrylamide–7 M urea gels and autoradiographed. On the right, two-dimensional projections of the DNA substrates are shown. Arrows indicate sites of hydroxyl radical cleavages and an asterisk denotes sites of endonucleolytic cleavages. Maxam–Gilbert sequencing reactions (G/A and C/T) are included in (B). The sites are numbered with respect to the scissile phosphates: cd, phosphate at the cleavage site of the coding strand and cd(+1) and cd(–2), phosphates located one nucleotide 3' to and two nucleotides 5' from the cd phosphate, respectively; nc, phosphate at the cleavage site of the non-coding strand; nc(–1) and nc(–2), phosphates located one and two nucleotides 5' to the nc phosphate, respectively.

the coding, or the non-coding strand (defined as the coding and non-coding strand in the natural substrate rRNA operon), complexed with the corresponding homing endonuclease, and incubated in the absence of divalent metal ion, or with Mg^{2+} , Fe^{2+} or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, before subjecting to denaturing polyacrylamide gel electrophoresis (Figure 3). Both *I-DmoI* and *I-PorI* exhibited normal endonucleolytic activity in the presence of Mg^{2+} and Fe^{2+} , producing products of the expected size (marked with an asterisk in Figure 3). Upon addition of Fe^{2+} and H_2O_2 , hydroxyl radical cleavages appeared at two major sites on each

strand (Figure 3). Hydroxyl radical cleavage generates 3'-phosphates, and these products are easily discriminated from the endonucleolytic cleavage which generates 5'-phosphates (Figure 3). The *I-DmoI*–DNA substrate was cleaved by hydroxyl radicals at the scissile phosphate on both the coding and non-coding strands (denoted as cd and nc, respectively, in Figure 3). In addition, strong cleavage was observed on both strands at phosphates two nucleotides further upstream from the scissile phosphates [cd(–2) and nc(–2), respectively, Figure 3A]. Weak, but reproducible cleavage was also observed at cd(–1) and cd(+1) on the coding strand. The *I-PorI* substrate was also cleaved at the scissile phosphates (cd and nc), in addition to cleavage at phosphates positioned one nucleotide downstream and upstream on the coding and non-coding strands [cd(+1) and nc(–1), respectively, Figure 3B]. Hydroxyl radical cleavage of the DNA was strictly dependent on the presence of the corresponding endonuclease (Figure 3), implying that the enzyme positioned the metal ions at the cleavage sites. In some instances the radical cleavages were induced by Fe^{2+} in the absence of H_2O_2 , possibly due to O_2 dissolved in the water substituting for H_2O_2 (see Figure 3A).

Metal ion competition experiments

In order to investigate the correlation between hydroxyl radical cleavage of the endonucleases and their DNA substrates more closely, Fe^{2+} was gradually replaced by titration with Ca^{2+} , which neither supports endonucleolytic activity nor can generate hydroxyl radicals. Ca^{2+} was added in concentrations ranging from 1 to 4 mM, and the effects on both endonucleolytic cleavages and hydroxyl radical cleavage at the four main sites in the protein and the DNA, were examined (Figure 4). In order to complex all labelled molecules, hydroxyl radical experiments on protein and DNA were done with a large excess of unlabelled protein or DNA ligand over the labelled DNA or protein partner (~10- to 25-fold the K_m value of the cleavage reaction). This ensures the presence of a stoichiometric protein–DNA complex in the assays. Furthermore, to avoid secondary effects, all assays were stopped early in the reaction, when cleavage rates at the individual sites were still constant. The results were quantified in an Instant Imager and semi-quantitative values averaged from at least three independent assays are summarized (see Figure 6, below).

These experiments showed that Ca^{2+} interfered with endonucleolytic cleavage and with Fe^{2+} binding to both DNA and protein, for both endonucleases (Figure 4; see also Figure 6). However, individual sites were affected to different degrees, allowing a distinction to be made between individual metal ions. Thus, for both *I-DmoI* and *I-PorI*, endonucleolytic cleavage of the non-coding strand was more affected by the presence of Ca^{2+} -ions than cleavage of the coding strand (Figure 4A). Moreover, assaying the *I-DmoI* protein, two hydroxyl radical cleavages on the protein (V39 and L113) and the DNA [nc and cd(–2)] were strongly reduced by Ca^{2+} ions, whereas the remaining two sites were less affected (Figure 4B left and middle panels, and C, left panels). *I-DmoI* was also tested at Ca^{2+} concentrations up to 20 mM, resulting in the gradual loss of all radical cleavage sites. This, however, gave no additional distinction between individual metal

ions (data not shown). For *I-PorI*, the presence of Ca^{2+} strongly reduced the hydroxyl radical cleavage at three sites on both protein (K8, V28 and L86) and DNA [nc, nc(-1) and cd(+1)], whereas a single site in the protein (V109) and the DNA (cd) was unaffected (Figure 4B and C, right panels).

Mutagenizing the LAGLIDADG motifs of *I-DmoI* and *I-PorI*

Hydroxyl radical cleavage of *I-DmoI* and *I-PorI* occurred within, and 10–15 amino acids after, each of the conserved LAGLIDADG motifs (Figure 2). This motif was shown earlier, by mutagenesis experiments on other LAGLIDADG-like endonucleases, to be important for catalysis but not for DNA binding (Gimble and Stephens, 1995; Henke *et al.*, 1995). Furthermore, all known LAGLIDADG endonucleases contain an acidic residue at the penultimate position of their LAGLIDADG motif (D21 and E117 in *I-DmoI* and D14 and E93 in *I-PorI*). Therefore, we reasoned that these amino acids are the most likely candidates for participating in the metal ion binding. To test this experimentally, these residues were changed to the corresponding amides, that are poor metal ion coordinators, in the first or the second LAGLIDADG motif of both *I-DmoI* (D21N and E117Q) and *I-PorI* (D14N and E93Q). The mutants were assayed for endonucleolytic cleavage activity (Figure 5A) and metal ion coordination to both protein (Figure 5B) and the DNA substrate (Figure 5C), using the Fe^{2+} -hydroxyl radical approach described above. The results averaged from at least three independent experiments are shown in Figure 6.

All mutations severely affected the endonucleolytic cleavage activity of both strands, although some residual activity was detected with the *I-PorI* D14N mutant (Figure 5A and 6). In agreement with reports for other homing enzymes (Gimble and Stephens, 1995; Henke *et al.*, 1995), the lack of cleavage was not a result of disabled DNA binding, since all four mutant proteins were found to bind specifically to the cognate DNA substrate using a proteinase footprinting approach described earlier (unpublished observation; Lykke-Andersen *et al.*, 1996). The mutants were all assayed for their ability to induce Fe^{2+} -hydroxyl radical cleavage of both the protein and the

Fig. 4. $\text{Fe}^{2+}/\text{Ca}^{2+}$ -competition experiments. (A) Endonucleolytic cleavage assays in the presence of Ca^{2+} as competitor. Experiments were performed at 65°C on a 25 bp *I-DmoI*-DNA substrate (left panel) or 30 bp *I-PorI* substrate (right panel), which were 5' end-labelled at the coding strand (C-lane) or the non-coding strand (N-lane) or both strands (all other lanes). Experiments were performed in the presence of 2 mM Fe^{2+} and 0, 1, 2 or 4 mM Ca^{2+} , as indicated. Controls of DNA substrate incubated with endonuclease in the absence of Fe^{2+} are included (-). The cleavage products were separated on 20% polyacrylamide-7 M urea gels and subjected to autoradiography. (B) Hydroxyl radical assays on N-terminally (HTG) and C-terminally (GTH) labelled *I-DmoI* and N-terminally (HTG) *I-PorI* proteins in the presence of Ca^{2+} as competitor. ^{32}P -end-labelled endonucleases were complexed with DNA substrate, and incubated with 2 mM Fe^{2+} and 0, 1, 2 or 4 mM CaCl_2 as indicated. Products were analysed on SDS-polyacrylamide gels and autoradiographed. Control samples of endonucleases incubated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ in the presence of 5 mM EDTA were included (-). The four main sites of hydroxyl radical cleavage are indicated by arrows to the right on each panel (see legend to Figure 2). (C) Hydroxyl radical cleavage of 25 and 30 bp DNA substrates of *I-DmoI* and *I-PorI*, respectively. The respective DNA substrates were 5' end-labelled on the coding or non-coding strand, complexed with *I-DmoI* (left panel) and *I-PorI* (right panel) and incubated with 2 mM $\text{Fe}^{2+}/1\%$ H_2O_2 and 0, 1, 2 or 4 mM CaCl_2 as indicated. Control samples, containing DNA incubated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ but no protein (-), were included. Products were denatured, separated on 20% polyacrylamide-7 M urea gels and autoradiographed. The major sites of hydroxyl radical cleavage are indicated with arrows (see legend to Figure 3). In (A), (B) and (C), cleavages highly affected by the presence of Ca^{2+} (>50% reduction at 2 mM) are indicated by ■.

DNA, and the results were compared with those of the unmutated proteins (Figures 5B and C and 6). The *I-DmoI* D21N mutant exhibited strongly reduced hydroxyl radical cleavage at two sites in the protein (V39 and K130) and the two scissile phosphates of the DNA substrate (cd and nc), whereas the other cuts were less affected. The *I-DmoI*

E117Q mutant showed strong reduction of hydroxyl radical cleavage only at K130 on the protein and at the coding strand scissile phosphate (cd). In *I-PorI*, the D14N mutation led to a reduced hydroxyl radical cleavage at the non-coding strand scissile phosphate (nc) with no significant reductions at the protein, while the E93Q mutant showed highly reduced cleavage at two sites in the protein (V109 and V28) and at the two scissile phosphates of the DNA (cd and nc). A double mutant of *I-PorI* (D14N/E93Q) was also tested; this was catalytically inactive and showed hydroxyl radical cleavage that was additive relative to the two single mutants (data not shown).

Discussion

DNA endonucleases require divalent metal ions to cleave DNA

Structural, biochemical and theoretical studies suggest that metal ions in DNases play a direct role in catalysis, although the exact function of the metal ions remains to be determined. The ability of the metal ion to act as a Lewis base may be important. Thus, a water molecule coordinated to the metal ion is deprotonated and the resulting hydroxyl ion can then perform a nucleophilic attack. A second potential function of the metal ions may be to stabilize the oxoanion leaving group of the cleavage reaction (Steitz and Steitz, 1993 and references therein). We have investigated two members of the LAGLIDADG-type homing endonuclease family, which resemble restriction endonucleases in several ways. They cleave both strands of the DNA within a specific nucleotide sequence yielding 5'-phosphates. However, in contrast to type II restriction endonucleases which recognize 4–8 base pair palindromic sequences, the homing endonucleases recognize more extended sequences of up to 20 base pairs which are generally non-palindromic, a property they share with type I restriction endonucleases. Another feature shared with restriction enzymes is the low degree of specificity for divalent metal ions. *I-DmoI* and *I-PorI* were

Fig. 5. Enzymatic activity and hydroxyl radical cleavage experiments using *I-DmoI* and *I-PorI* mutants. Endonucleases mutated in the penultimate acidic residue of either the first or the second LAGLIDADG motif of *I-DmoI* (D21N and E117Q) and *I-PorI* (D14N and E93Q) were tested for endonucleolytic DNA cleavage activity and $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -induced radical cleavage of protein and DNA substrate. (A) Endonuclease activity of wild-type (wt) and mutant *I-DmoI* (D21N, E117Q; left panel) and *I-PorI* (D14N, E93Q; right panel), determined as described in the legend to Figure 1, but in the presence of 10 mM MnCl_2 and with the 25 bp and 30 bp DNA substrates for, respectively, *I-DmoI* and *I-PorI*. Experiments with 10 mM MgCl_2 gave similar results (not shown). Control samples incubated in the absence of endonuclease (–) are included. Arrows indicate cleavage at the coding (cd) and non-coding strands (nc). The 30 bp substrate used with *I-PorI* (right panel) was contaminated with smaller fragments (28–29 bp), giving rise to smaller cleavage products, that are indicated by asterisks. (B) Hydroxyl radical cleavage of wild-type and mutant endonucleases, N-terminally (HTG) or C-terminally (GTH) labelled and assayed as described in the legend to Figure 2. Control samples of labelled endonucleases incubated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ in the presence of EDTA were included (–). The four main sites of hydroxyl radical cleavage are indicated by arrows to the right in each panel (see legend to Figure 2). (C) Fe^{2+} -hydroxyl radical cleavage on the 25 bp and 30 bp DNA substrates of *I-DmoI* and *I-PorI*, respectively, in the presence of wild-type and mutant endonucleases, assayed as described in the legend to Figure 3. Major cleavage sites are shown by arrows. Control samples of labelled DNA incubated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ in the absence of endonuclease (–) are included.

Fig. 6. Summary of hydroxyl radical and endonucleolytic cleavage in Ca^{2+} competition and mutagenic experiments for (A) *I-DmoI* and (B) *I-PorI*. Band intensities of Fe^{2+} -hydroxyl radical cleavage on the proteins ($\text{Fe}/\text{H}_2\text{O}_2 \rightarrow \text{Protein}$), the DNA substrate ($\text{Fe}/\text{H}_2\text{O}_2 \rightarrow \text{DNA}$) and the endonucleolytic cleavage of the DNA substrate (Enzymatic Cleavage), from at least three independent experiments of which one is illustrated in Figures 4 and 5, were quantified on an Instant Imager, and the averaged values are indicated semi-quantitatively below schematic representations of the affected sites on the proteins (see Figure 2) and DNA substrates (see Figure 3). The values correspond to band intensities of: +++, 80–100%; ++, 50–80%; +, 20–50%; (+), <20% and –, 0% of that of the wild-type cleavage with no competitor (wt). Values are calculated for wild-type endonucleases in the presence of 2 mM Ca^{2+} competitor (Ca^{2+}), and for the experiments with the *I-DmoI* D21N and E117Q mutants and the *I-PorI* D14N and E93Q mutants as indicated. Sites of Fe^{2+} -induced hydroxyl radical cleavages are given by arrows on schematic representations of the proteins and DNA substrates.

active in the presence of Mn^{2+} , Fe^{2+} , Mg^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} , whereas Cu^{2+} , Ca^{2+} and Ba^{2+} ions were unable to support catalysis. A similar pattern was observed for *EcoRI* and *EcoRV* (Vipond *et al.*, 1995). That Ca^{2+} is unable to function as a metal ion cofactor in the cleavage is somewhat surprising, since Mg^{2+} and Ca^{2+} are chemically very similar, although Ca^{2+} may fail to enter the catalytic site due to its larger ionic diameter. This is unlikely, however, since Ca^{2+} inhibited both endonucleolytic cleavage of DNA by *I-DmoI* and *I-PorI* and also Fe^{2+} -induced hydroxyl radical cleavages at protein and DNA substrate. Another possibility is that Ca^{2+} ions disturb the overall structure of the enzyme, although this is also rendered unlikely by the observation that Ca^{2+} only efficiently inhibited enzymatic cleavage of one strand, for both endonucleases. Furthermore, the simple change in ionic strength does not lead to structural changes, since increasing the concentration of monovalent cations (K^+) above the ionic strength of the highest Ca^{2+} concentration inhibited neither endonucleolytic cleavage nor Fe^{2+} -induced hydroxyl radical cleavage. Moreover, increasing Mg^{2+} concentrations increased the endonucleolytic cleavage (data not shown). A third explanation is that Ca^{2+} binds to the active site of *I-DmoI* and *I-PorI* but fails to support the catalytic reaction. All the data presented here are consistent with this model; in particular, the observation that Ca^{2+} inhibits a Fe^{2+} -catalysed cleavage reaction suggests that it competes directly for Fe^{2+} binding to the

proteins. An attempt was made to establish that Ca^{2+} binds directly at the catalytic centre of each endonuclease, by performing competition experiments with Ca^{2+} and Mg^{2+} . However, since no Michaelis–Menten kinetics were observed with respect to Mg^{2+} (data not shown), a clear distinction between competitive and non-competitive binding could not be made. Interestingly, Ca^{2+} seems to be an acceptable cofactor for the non-LAGLIDADG nuclear homing endonuclease *I-PpoI*, indicating a different geometry of the catalytic site in that protein (Wittmayer and Raines, 1996). However, which metal ion is used *in vivo* remains an unanswered question; possibly the enzyme uses whichever suitable divalent metal ion is available.

Mapping metal binding sites

To map the divalent metal ion binding sites of the two archaeal homing endonucleases, *I-DmoI* and *I-PorI*, we employed Fenton chemistry, involving the generation of reactive intermediates from H_2O_2 in the vicinity of Fe^{2+} (for review, see Price and Tullins, 1992). This method has previously been applied successfully to map divalent metal binding sites of *E.coli* glutamine synthetase (Farber and Levine, 1986), the Tet repressor (Ettner *et al.*, 1995), pigeon liver malic enzyme (Wei *et al.*, 1995) and *E.coli* RNA polymerase (Zaychikov *et al.*, 1996). In the Tet repressor study, a close correlation between cleavage efficiency and the distance of the cleaved bonds to Mg^{2+}

in the crystal structure of the Tet repressor was found, implying that the reactive intermediates are very short-lived compared with the speed of diffusion, and that the cleavage reaction is a valid measurement for the proximity of the Fe^{2+} ion (Ettner *et al.*, 1995). The mechanism of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -induced cleavage has been discussed (Rana and Meares, 1991; Price and Tullins, 1992; Platis *et al.*, 1993; Ettner *et al.*, 1995). It is generally assumed that cleavage mainly is a result of hydroxyl radical attack. However, some evidence also points towards a non-radical mechanism involving a nuclear attack of an activated oxo intermediate (Rana and Meares, 1991). In our investigations, cleavage of both the DNA and the protein were strictly Fe^{2+} -dependent but, in some experiments, H_2O_2 was dispensable (e.g. Figure 2A). This could be explained by a non-radical cleavage mechanism in which nearby hydroxyl groups, coordinated by the ferrous ion, are involved in a nucleophilic attack or, perhaps more likely, by the presence of soluble O_2 -forming radicals (Price and Tullins, 1992).

Both the homing enzymes and their DNA substrates were specifically cleaved at four major positions in the presence of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ (Figures 2 and 3). The DNA cleavages were dependent on the presence of the homing enzymes and the protein cleavages were strongly enhanced by the presence of the DNA substrates. This suggests that the metal ions are coordinated primarily to the protein, but stabilized by a phosphodiester group of the DNA substrate as has been shown for the *EcoRV*-DNA complex (Kostrewa and Winkler, 1995). Alternatively, the DNA may induce perturbations in the protein structure, making the adjacent amino acids more susceptible to cleavage by free Fe^{2+} ions. Several observations are compatible with the Fe^{2+} ion(s) contributing to the function of the catalytic site. Firstly, the endonucleases were highly active in the presence of Fe^{2+} ions. Secondly, the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ -dependent cleavages occurred at or near the scissile phosphates, and within and immediately after the LAGLIDADG regions of the enzymes, that constitute a conserved and functionally important region of these homing endonucleases (Gimble and Stephens, 1995; Henke *et al.*, 1995). Thirdly, *I-DmoI* and *I-PorI* both bind specifically to the DNA substrates in the absence of divalent metal ions (Lykke-Andersen *et al.*, 1996). Finally, a strong correlation exists between enzymatic activity and Fe^{2+} binding under a variety of experimental conditions (see below).

Locating individual metal ions in the endonuclease-DNA complex

To localize individual Fe^{2+} ions within the protein and DNA structures, a combination of Ca^{2+} competition and mutagenesis experiments was used. The former exploit the observation that Ca^{2+} ions cannot induce endonucleolytic cleavage of the DNA. Titration of Ca^{2+} ions into a reaction containing a fixed concentration of Fe^{2+} produced unequal competition for enzymatic cleavage of the two DNA strands. For both *I-DmoI* and *I-PorI*, enzymatic cleavage of the coding strands was least sensitive to addition of Ca^{2+} , implying that more than one metal ion is responsible for catalytic cleavage and that the Fe^{2+} responsible for cleaving the coding strand is more tightly bound than the non-coding strand-cleaving Fe^{2+} . This resembles the observation for the homing endonuclease, *I-CpaII*,

Fig. 7. A schematic model for metal ion coordination sites within the catalytic centre of *I-DmoI*. One helical turn of DNA that contains the endonucleolytic cleavage site for *I-DmoI* is projected in two dimensions and non-coding (nc) and coding strands (cd) are shown. Nucleosides are denoted by dots and the scissile phosphates (P) are boxed. Bold and thin arrows indicate strong and weak hydroxyl radical cleavage sites, respectively. The identities of the amino acids cleaved by hydroxyl radicals are approximate and may vary by ± 2 amino acids. Straight lines indicate that binding of this Fe^{2+} ion is dependent on the identity of the connected amino acid. Ca^S and Ca^R indicate whether the position of this ion is sensitive or resistant to Ca^{2+} titration, respectively. The spatial position of the Fe^{2+} and the structure of the polypeptide chain is drawn arbitrarily.

encoded by the chloroplast rRNA intron of *Chlamydomonas pallidostigmatica*, for which cleavage of the non-coding strand was impaired more at low Mg^{2+} than cleavage of the coding strand (Turmel *et al.*, 1995). In contrast, DNA cleavage by restriction endonucleases *EcoRV* and *SaII* shows no strand specificity at low Mg^{2+} concentration, reflecting that each subunit in the homodimeric complex has similar affinities for metal ions (Halford and Goodall, 1988).

When combining the results from the Ca^{2+} competition and mutagenesis experiments, the differential behaviour of individual Fe^{2+} ions indicates that the *I-DmoI*-DNA substrate complex contains a minimum of four metal ions, with the metal ions near L14, V39, L113 and K130 of the protein positioned at the nc(-2), nc, cd(-2) and cd phosphates of the DNA substrate, respectively (Figures 6 and 7). Thus, the V39/nc and L113/cd(-2) metal sites are more sensitive to Ca^{2+} than the other two sites, the K130/cd site is sensitive to mutation at D21 and the V39/nc site is sensitive to mutations at both D21 and E117 (Figures 6 and 7). *I-PorI* may also contain four metal ions. Here, the metal ions near V28 and V109 are probably positioned at the nc and cd phosphates, respectively, whereas the metal ions at K8 and L86 of the protein and nc(-1) and cd(+1) of the DNA cannot be discriminated by our data (Figure 6).

Taken together, the data are consistent with a model where each endonuclease cleaves its DNA substrate as a monomeric enzyme with four metal ions positioned at, or near, the catalytic centre. The metal ions coordinated to the scissile phosphates of the non-coding and the coding strand map 10–15 amino acids C-terminal to the first and second LAGLIDADG motifs of the proteins, respectively, and the identity of these metal ions is decisive for

endonucleolytic cleavage at the corresponding phosphate, as seen from the Ca^{2+} titrations. Moreover, judging from the mutational data, the conserved penultimate acidic residues of the LAGLIDADG motifs are probably involved in coordinating the metal ions at the scissile phosphates. Two other metal ions, which are placed 1–2 nucleotides from the cleavage site, are not coordinated by these amino acids but are positioned near the N-terminus of the LAGLIDADG motifs. Although we cannot conclude that all the metal ions have a specific role in the catalytic reaction, it is an attractive hypothesis that the monomeric endonuclease coordinates two metal ions at each of the two active sites, where one metal ion activates the attacking hydroxyl group, while the other ion stabilizes the oxyanion leaving group as shown for a number of other DNases, and proposed for catalytic RNAs (Steitz and Steitz, 1993).

Materials and methods

Vectors and substrates

Vectors for expression of GST–endonuclease fusion proteins in *E. coli* pET–HTG–*DmoI*, pGEX–GTH–*DmoI*, pET–HTG–*PorI* and pGEX–GTH–*PorI* were described earlier (Lykke-Andersen *et al.*, 1996). The plasmids pUC–*Dmuc* and pUC–*Pisl*, used in the cleavage assays, are pUC19 derivatives containing 245 and 205 bp inserts with cleavage sites for I–*DmoI* and I–*PorI*, respectively (Lykke-Andersen *et al.*, 1996). 25 bp DNA substrates were prepared by annealing the oligodeoxynucleotides 5′-GCCTTGCCGGGTAAGTTCGCG-CGCG and 5′-GCGAGCCCGT-AAGGGTGTGTACGGG with their complementary sequences, for I–*DmoI* and I–*PorI* respectively (Lykke-Andersen *et al.*, 1996) and a 30 bp substrate for I–*PorI* was prepared by annealing the oligodeoxynucleotide 5′-CGCGAGCCCGTAAGGGTGTGTACGGGGGCT with its complementary sequence.

Endonuclease assays

Plasmids pUC–*Pisl* and pUC–*Dmuc* were cleaved with *PvuII*, dephosphorylated with calf intestinal alkaline phosphatase (Boehringer), and 5′-end-labelled using T4 polynucleotide kinase (Amersham) and [γ - ^{32}P]ATP (Amersham). About 10 fmol I–*DmoI* and I–*PorI* were mixed with ~50 c.p.s. ^{32}P -labelled substrate (~50 fmol) in 10 μl binding buffer (25 mM HEPES–KOH, pH 8.0, 50 mM KCl, 5 mM dithiothreitol, 2% glycerol, 0.05% Triton X-100), and the mixture was overlaid with mineral oil. A divalent cation chloride was added at 1 mM, and reaction mixtures were incubated at 65°C (I–*DmoI*) or 80°C (I–*PorI*) for 10 min. DNA was precipitated with ethanol, redissolved in formamide, denatured at 95°C for 2 min and run on a 5% polyacrylamide–7 M urea gel.

Determination of metal ion binding sites on proteins and DNA

In order to determine metal binding sites on I–*DmoI* and I–*PorI*, N- and C-terminally ^{32}P -labelled endonucleases were generated from vectors pET–HTG–*DmoI*, pGEX–GTH–*DmoI*, pET–HTG–*PorI* and pGEX–GTH–*PorI* as described earlier (Lykke-Andersen *et al.*, 1996), and ~200 c.p.s. (~1 pmol) labelled endonuclease was incubated with 20 pmol 25 bp DNA substrate in 20 μl binding buffer at 65°C for 5 min. Samples were cooled to 50°C and 2 μl of 20 mM $\text{Fe}(\text{NH}_4)_2\text{SO}_4$, 1% H_2O_2 and 100 mM sodium ascorbate was added. In Ca^{2+} -competition experiments, CaCl_2 was added at different concentrations together with the $\text{Fe}(\text{NH}_4)_2\text{SO}_4$. After 5 min of incubation, the reaction was stopped by adding 10 μl 100 mM Tris base, 15% glycerol, 1.5% sodium dodecylsulphate, 200 mM dithiothreitol, 20 mM EDTA and 100 mM thiourea and the samples were run on a large 7% stacking/20% separation polyacrylamide–SDS–Tricine gel, which was then subjected to autoradiography. Gels of Ca^{2+} -competition experiments were quantified on an Instant Imager (Packard, Meriden, USA).

To determine the metal coordination sites on the DNA substrates, 25-mer oligodeoxynucleotides encompassing the cleavage sites for I–*DmoI* and I–*PorI* were 5′ end-labelled using T4 polynucleotide kinase (Amersham) and [γ - ^{32}P]ATP (Amersham), and complexed with the complementary unlabelled 25-mer by mixing at 0.1 μM in 10 mM HEPES–KOH, pH 8.0, 100 mM KCl, heating at 95°C for 2 min, followed by incubation at 65°C for 5 min and slowly cooling to room temperature.

In some experiments, 30-mer oligonucleotides were used for I–*PorI*. Approximately 100 c.p.s. substrate (~0.1 pmol) was mixed with ~1 pmol purified I–*DmoI* and I–*PorI*, expressed from pET–HTG derivatives (Lykke-Andersen *et al.*, 1996), in 10 μl binding buffer at 65°C for 5 min. Samples were cooled to 50°C and 1 μl of a mixture of 20 mM $\text{Fe}(\text{NH}_4)_2\text{SO}_4$, 1% H_2O_2 , 100 mM ascorbate was added. In Ca^{2+} -competition experiments, CaCl_2 was added in different concentrations together with the $\text{Fe}(\text{NH}_4)_2\text{SO}_4$. After 5 min incubation the reaction was stopped by addition of 40 μl of 0.3 M Na acetate, pH 6.0, 5 mM EDTA, 100 mM thiourea, 0.1 mg/ml tRNA and 125 μl ethanol. DNA was precipitated, washed in 80% ethanol, redissolved in formamide, denatured at 95°C for 2 min and run on 20% polyacrylamide–7 M urea gels which were then subjected to autoradiography. Gels of Ca^{2+} -competition and endonuclease mutant experiments were also quantified on an Instant Imager (Packard, Meriden, USA).

Mutagenesis of I–*DmoI* and I–*PorI*

The open reading frames of I–*DmoI* and I–*PorI* were excised from pET–HTG–*DmoI* and pET–HTG–*PorI* vectors (Lykke-Andersen *et al.*, 1996) using *Bam*HI and *Eco*RI, and ligated with *Bam*HI–*Eco*RI-cleaved M13mp19. Site-directed mutagenesis was performed according to the method of Kunkel *et al.* (1987). For I–*DmoI*, oligonucleotides 5′-GTAAAGTCTCCATTACCTATTATCAATCC and 5′-GGTTTATCTCCTTGAGCTACAT-ATAGCCC (position of mutation underlined) were used to mutagenize the first and second LAGLIDADG motif, respectively. For I–*PorI* an internal *Eco*RI site in the open reading frame was first destroyed by a silent mutation, changing one glutamate codon (GAA) into another (GAG), using the oligodeoxynucleotide 5′-CTCGAGG-AACTCCTTAGA. The resulting *Eco*RI-minus clone was then used to mutagenize the LAGLIDADG motifs, using oligodeoxynucleotides 5′-AACATAGCCATTA-AAAGCGCT for the first and 5′-CACATT-CCCTTGGGCATCTATT for the second motif. The mutated open reading frames were cut out using *Bam*HI and *Eco*RI and inserted into pET–HTG and pGEX–GTH vectors (Jensen *et al.*, 1995b) and the proteins were expressed and purified as described earlier for the wild-type protein (Lykke-Andersen *et al.*, 1996).

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