Mutational analysis of human DNase I at the DNA binding interface: Implications for DNA recognition, catalysis, and metal ion dependence

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Abstract

Human deoxyribonuclease I (DNase I), an enzyme used to treat cystic fibrosis patients, has been systematically analyzed by site-directed mutagenesis of residues at the DNA binding interface. Crystal structures of bovine DNase I complexed with two different oligonucleotides have implicated the participation of over 20 amino acids in catalysis or DNA recognition. These residues have been classified into four groups based on the characterization of over 80 human DNase I variants. Mutations at any of the four catalytic amino acids His 134, His 252, Glu 78, and Asp 212 drastically reduced the hydrolytic activity of DNase I. Replacing the three putative divalent metal ion-coordinating residues Glu 39, Asp 168, or Asp 251 led to inactive variants. Amino acids Gln 9, Arg 41, Tyr 76, Arg 111, Asn 170, Tyr 175, and Tyr 211 were also critical for activity, presumably because of their close proximity to the active site, while more peripheral DNA interactions stemming from 13 other positions were of minimal significance. The relative importance of these 27 positions is consistent with evolutionary relationships among DNase I across different species, DNase I-like proteins, and bacterial sphingomyelinases, suggesting a fingerprint for a family of DNase I-like proteins. Furthermore, we found no evidence for a second active site that had been previously implicated in Mn^{2+} -dependent DNA degradation. Finally, we correlated our mutational analysis of human DNase I to that of bovine DNase I with respect to their specific activity and dependence on divalent metal ions.

Keywords: DNase I; protein-DNA interactions; site-directed mutagenesis; structure-function analysis

Human deoxyribonuclease (DNase I), a pancreatic endonuclease that catalyzes the hydrolysis of double-stranded DNA, is currently used for the treatment of pulmonary complications in cystic fibrosis patients (Ramsey, 1996). The recombinant human enzyme is inhaled into the airways where it degrades DNA to lower molecular weight forms, thus reducing the viscoelasticity of CF sputum and improving lung function (Fuchs et al., 1994; Ramsey et al., 1993). In addition, the use of DNase I in a murine model of systemic lupus erythematosus (SLE) has been investigated with encouraging results (Macanovic et al., 1996). The pharmacological and clinical significance of DNase I has led us to recently engineer actin-resistant

variants of the human enzyme that are no longer inhibited by actin (Ulmer et al., 1996) as well as hyperactive variants that alter the functional mechanism of DNA cleavage (Pan & Lazarus, 1997, 1998).

DNase I from bovine pancreas has been studied extensively (Laskowski, 1971; Moore, 1981; Suck, 1994). The enzyme requires divalent metal ions and displays optimal activity in the presence of Ca^{2+} and Mg^{2+} or Ca^{2+} and Mn^{2+} (Price, 1975). It degrades DNA nonspecifically by a single-stranded nicking mechanism in the presence of Mg^{2+} ions while double-stranded cutting has been observed in the presence of Mn^{2+} or Mg^{2+} , and Ca^{2+} (Campbell & Jackson, 1980; Melgar & Goldthwait, 1968). The crystal structure of bovine DNase I refined to 2 Å resolution shows two Ca^{2+} ions that stabilize surface loops as well as an additional metal ion binding site at the active site (Oefner & Suck, 1986). The structures of the enzyme complexed to a nicked DNA octamer (Octa I) solved at 2 Å (Lahm & Suck, 1991) and an intact DNA octamer (Octa V) at 2.3 Å (Weston et al., 1992) have implicated a general acid-base mechanism for the hydrolysis of the phosphodiester bond, in which His 134 acts as the general acid while His 252 serves as the general base (Fig. 1). Both histidine residues are hydrogen bonded to carboxylate groups (Glu 78 and Asp 212, respectively), thus raising their pK_a values (Perutz et al., 1985). The required metal ion at the active site is coordinated to the scissile phosphate group, Glu 39, and pos-

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Abbreviations: DNase I, deoxyribonuclease I; CF, cystic fibrosis; SLE, systemic lupus erythematosus; EDTA, ethylenediamine tetraacetic acid; TBE, tris-borate EDTA; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SMase, sphingomyelinase. One or three letter codes are used to represent naturally occurring L-amino acids. When referring to mutants the wild-type amino acid is followed by the residue number and the new amino acid as in H134Q; DNase I variants with multiple mutations are denoted with a colon between the individual mutations such as H134Q:H252Q.

Fig. 1. Model of the human DNase I-DNA complex. Twenty-two residues at the DNA binding interface of human DNase I (white) that were altered are labeled and colored according to their functional roles: yellow for catalysis, magenta for metal ion binding, red for crucial DNA contacts, and blue for non-critical DNA contacts. The DNA is colored in light green with the scissile bond in the putative second active site highlighted in darker green on the left and the scissile bond in the actual active site in the darkest green on the right. The model was derived by superposition of human DNase I (Wolf et al., 1995) with the bovine DNase I-octamer complex (Weston et al., 1992). The figure was made using MidasPlus (Ferrin et al., 1988).

sibly Asp 168 and Asp 251. The protein-DNA complex is stabilized by a network of 15 direct DNA contacts contributed by residues Arg 9, Glu 13, Thr 14, Arg 41, Ser 43, His 44, Asn 74, Ser 75, Tyr 76, Arg 111, Asn 170, Tyr 175, Ser 206, Thr 207, and Tyr 211 (Lahm & Suck, 1991; Weston et al., 1992).

Despite extensive biochemical characterization of the bovine enzyme, only recently have site-directed mutagenesis studies on the functional importance of DNase I residues involved in DNA recognition and catalysis been addressed (Doherty et al., 1995; Jones et al., 1996; Warren et al., 1997). Compared to bovine DNase I, the clinically more relevant human enzyme shares 78% identity (Shak et al., 1990) and is 2.4-fold less active (Sinicropi et al., 1994). In the present work, we have systematically analyzed the functional significance of each one of the above 22 residues and five additional positions in the DNA-binding interface by constructing site-directed human DNase I mutants, expressing them in human 293 cells, and characterizing the cell culture media in two activity-based assays. We propose the classification of these residues into four groups based on their functional roles in catalysis,

metal ion coordination, critical DNA contacts surrounding the active center, and more peripheral DNA interactions that contribute little to activity. The first three groups were highly conserved among DNase I from different species, DNase I-like proteins, and another phosphodiesterase, bacterial sphingomyelinase. Furthermore, we have compared the DNA cleavage activities of human and bovine pancreatic DNase I in the presence of Ca^{2+} , Mg^{2+} , and Mn^{2+} ions. Although the metal ion dependence profiles for these two enzymes are similar, their differences allowed us to speculate on the importance of various residues that contact DNA.

Results

Active site residues

The two catalytic histidines at positions 134 and 252 and their hydrogen-bond pairs Glu 78 and Asp 212 are crucial for the functional activity of human DNase I, as mutations at these positions dramatically reduced the DNA degradation activity. In a previous

Fig. 2. Overall plasmid nicking activities of human DNase I mutations of (A) the catalytic and (B) the critical DNA recognition residues. The supercoiled plasmid digestion assay was performed in the presence of Mn^{2+} ions with the exception of Q9A which was done in Mg^{2+} and Ca^{2+} ions. The asterisk (*) denotes the upper limit on the activities of some of the variants due to their poor expression in the culture media.

study (Ulmer et al., 1996), we could not detect any significant activity for selected mutants (H134A, H134Q, H252A, H252Q, E78A, and D212A) using a relatively insensitive DNA-methyl green assay, which measures a decrease of A_{620} as the methyl green dye is released from hydrolyzed DNA. Similar findings were obtained here with three additional mutants, H134C, H252C, and E78Q (data not shown). In order to quantify the activity of these mutants and assess the relative importance of the catalytic residues, we utilized a more sensitive assay involving digestion of supercoiled plasmid. The plasmid digestion assay follows the conversion of plasmid pBR322 DNA from a supercoiled substrate to a relaxed circle as a result of a single nick on one of the two DNA strands and subsequently to linear DNA if it is nicked on the other strand at the same position. The overall plasmid nicking activity measures the disappearance of the supercoiled substrate; the linear to relaxed product ratio sheds light upon the mechanism of DNA cleavage. We found that alanine mutations at the two histidine positions tend to have a more deleterious effect than those at the carboxylate positions (Fig. 2A). In addition, an alanine substit-

ution for His 252 results in ~10-fold lower DNase I activity relative to the more conservative glutamine replacement. The digestion activity of the combination variant H134Q:H252Q is ~170,000-fold lower than that of wild type (Fig. 3). Mutations at the two histidines did not significantly change the linear to relaxed product ratio in the supercoiled plasmid digestion assay. On the other hand, although the carboxylate mutations E78A, E78Q, and D212A do not lower the overall plasmid nicking activity as much as the histidine mutations, their plasmid digestion pattern exhibited a much lower linear to relaxed product ratio when compared to wild type (data not shown).

Metal ion binding residues

The position of a divalent metal ion at the active site has been implicated by soaking DNase I crystals in a solution of deoxythymidine-3', 5'-diphosphate (pdTp) in the presence of Ca^{2+} (Suck & Oefner, 1986). The crystal structures of DNA-DNase I complexes (Lahm & Suck, 1991; Weston et al., 1992), together with that of Ca-pdTp-DNase I complex, suggest that the metal ion stabilizes the pentavalent transition state of the scissile phosphate group. Three carboxylate residues—Glu 39, Asp 168, and Asp 251—near the scissile bond may serve as ligands for the metal ion (Fig. 1). To test the importance of these residues as well as their potential role in metal ion binding, we constructed individual alanine, lysine, and arginine replacement variants of human DNase I. A positively charged amino group of a lysine or an arginine stemming from any one of the these three positions might replace the role of the metal ion in stabilizing the negatively charged scissile phosphate. However, all nine of these mutations resulted in no detectable activities in the DNA-methyl green assay; activities in the supercoiled plasmid digestion assay in the presence of Mg^{2+} and Ca^{2+} ions were at least 10^4 times lower than that of wild type. Furthermore, no supercoiled plasmid digestion activities were observed for these variants in the absence of metal ions even when they were used in concentrations as high as 10 μ g/mL. We reason that Glu 39, Asp 168, and Asp 251 are critical for activity due to their ability to coordinate one or more divalent metal ions at the active center. It is unlikely that these residues provide any direct contact with DNA based on structural and electrostatic considerations. In addition, most Mg^{2+} and Ca^{2+} coordinating ligands stem from aspartic or glutamic residues. Moreover, the attempt to replace the carboxylate-metal ion complex with an arginine or lysine was unsuccessful, suggesting that the functional role of the

Fig. 3. Plasmid DNA digestion by wild-type human DNase I and the catalytically inactive mutant H134Q:H252Q. Supercoiled pBR322 substrate with a low level of relaxed circle background (first lane, "C" for control) was incubated with 0.5 ng/mL of wild type or 3800 ng/mL of the H134Q:H252Q DNase I for 1 to 64 min in the presence of Mg^{2+} and Ca^{2+} ions. The linear product (L) ran in between the slower moving relaxed product (R) and the faster moving supercoiled substrate (S).

metal ion depends not only on its positive charge, but also on its precise orientation relative to the scissile bond and its specific chemical properties.

Critical DNA contacts

An alanine scan of the 15 DNase I positions that make direct contacts with DNA as observed in the two crystal structures of DNA-DNase I complexes (Lahm & Suck, 1991; Weston et al., 1992) revealed key contacts by seven residues that surround the catalytic center (Figs. 1, 2B). Alanine substitutions at five of these positions (Gln 9, Tyr 76, Arg 111, Asn 170, and Tyr 211) yielded variants that showed no detectable activities in the DNA-methyl green assay; R41A and Y175A exhibited 65-fold and 16-fold lower activities than wild type, respectively. Again, the more sensitive supercoiled plasmid digestion assay allowed us to measure some activity with each of these variants except one (Fig. 2B). The most severe effects come from replacements at Asn 170 and Arg 111. Asn 170 is proximal to the two catalytic histidines and can form a hydrogen bond with the scissile phosphate group; Arg 111 is also nearby and forms a salt bridge with the phosphate next to the scissile bond (Fig. 1). Contributions by Gln 9, Arg 41, Tyr 76, and Tyr 211 contacts are also quite significant since alanine substitutions at these positions resulted in enzymes with at least 60-fold lower activities than wild type. The Tyr 175 interaction is less important with Y175A being 15-fold less active than wild type, perhaps because it is farther away from the active site than the other six residues.

Putative second active site

In the crystal structure of the complex between the bovine pancreatic DNase I and d(GCGATCGC)₂ (Octa I), the nicked octamer duplex lost a dinucleotide (pGpC) from the 3' end of one strand despite the presence of EDTA (Lahm & Suck, 1991). When the octamer-DNase I co-crystals were soaked with Mn²⁺, but not Ca²⁺ or Mg²⁺, a second nick was observed four base-pairs away in the opposite strand (Fig. 1). Based upon these observations, the presence of a secondary active site near Ser 43, which is only activated by Mn²⁺, was suggested (Lahm & Suck, 1991). This Mn²⁺-dependent secondary active site could explain the shift from single-stranded scission to a double-stranded cutting mechanism when the divalent cation is changed from Mg²⁺ to Mn²⁺ (Campbell & Jackson, 1980; Melgar & Goldthwait, 1968). If this Mn²⁺-dependent active site indeed exists, mutations at this region of DNase I should reduce the linear to relaxed product ratio in the supercoiled plasmid digestion assay in the presence of Mn²⁺ but not Mg²⁺ ions.

To investigate the existence of this putative secondary active site, we mutagenized human DNase I residues in this region and compared their supercoiled plasmid digestion pattern with that of wild type in the presence of Mg²⁺ and Mn²⁺ ions. As in bovine DNase I (Price, 1975), human DNase I exhibits much higher overall supercoiled plasmid digestion activity and a greater linear to relaxed product ratio in the presence of Mn²⁺ than Mg²⁺ (Fig. 4A). A close examination of the Octa I-DNase I complex shows that the side-chain hydroxyl group of Ser 43 is near the scissile phosphate group of the putative secondary site and may serve as the nucleophile necessary for catalysis (Fig. 1). Replacing Ser 43 of the human enzyme with an alanine had no effect either on the overall activity or the linear to relaxed product ratio in the presence of either metal ion. With the exception of R41A, other mutations in

Fig. 4. Plasmid DNA digestion by human DNase I variants at the second putative active site. **A:** Supercoiled pBR322 substrate with a low level of relaxed circle background (first lane, "C" for control) was treated with 1 ng/mL of wild type or S43A DNase I for 5 to 20 min in the presence of Mg²⁺ (top panel) or Mn²⁺ ions (bottom panel). **B:** Supercoiled pBR322 was treated with 0.1 ng/mL of wild type, T14A, S43A, or H44A, or 5 ng/mL R41A for 1, 3, and 10 min in the presence of Mn²⁺ ions. The linear product (L), relaxed product (R), and supercoiled substrate (S) are denoted.

this putative second site, including E13A, E13R, T14A, H44A, and S43A:H44Q, showed no significant difference on the supercoiled plasmid digestion pattern from the wild-type protein in the presence of either Mg²⁺ or Mn²⁺ ions (data not shown and Fig. 4B). The fact that the activity of R41A is ~50-fold lower than wild type in the presence of Mg²⁺ (data not shown) or Mg²⁺ and Ca²⁺ (Fig. 2B) as well as Mn²⁺ (Fig. 4B) suggests that Arg 41 is not involved in this putative second site. Therefore, we conclude that a Mn²⁺-dependent secondary active site does not exist, at least in human DNase I.

Peripheral DNA contacts

Recently, we engineered hyperactive human DNase I by introducing positively charged amino acids at eleven DNase I positions that can interact favorably with the proximal negatively charged phosphate groups of the DNA but are distal to the active site (Pan & Lazarus, 1997). In the present work, we characterized these hyperactive positions in more detail by making additional mutations and testing them in the presence of both Mg²⁺ and Ca²⁺ ions. Analysis of Glu 9 in the human nuclease suggests that it makes a critical contact with DNA via hydrogen bonding with its amido group and van der Waals interaction with its γ -methylene group. The loss of both types of interactions in Q9A resulted in a drastic decrease in activity in both the DNA-methyl green (Fig. 5A) and the supercoiled plasmid digestion assay (Fig. 2B); retention of the hydrophobic interactions in Q9M yielded a more modest reduction of fourfold to fivefold in activity in both assays. The activity of Q9E was also reduced fourfold to fivefold, which is likely due to the introduction of a negative charge close to the negatively charged DNA phosphate backbone. Finally, the lysine substitution increased the methyl green activity slightly over the wild type while

Fig. 5. DNA-methyl green activity of human DNase I variants. The assay was performed in the presence of both Mg^{2+} and Ca^{2+} ions. The results are presented as percent activity of wild type.

the arginine replacement elevated the activity by 1.7-fold, which may be explained by an attractive electrostatic effect (Pan & Lazarus, 1997).

The same electrostatic interactions explain the activity of most variants at positions 13, 44, and 74. The replacement of the negatively charged Glu 13 with any non-negative amino acid resulted in an increased activity in the DNA methyl green assay (Fig. 5A). In the supercoiled plasmid digestion assay, E13D has virtually the same activity as wild type while that of E13Q is approximately twofold higher (data not shown). Likewise, the negatively charged amino acid substitutions at position 44 lowered the methyl green activity whereas the arginine replacement increased activity by 30% (Fig. 5B); neutral mutations had no effect, with the exception of asparagine. The plasmid digestion assay also showed an approximately twofold decrease by H44D, H44E, and H44N and no change in activity for H44A. The lower specific activity of H44N can be explained by the fact that this mutation introduces a new N-linked glycosylation site which could interfere with DNA binding. All 19 possible amino acid substitutions were introduced at position 74 due to the tendency of the wild-type asparagine to deaminate (Cacia et al., 1993). Mutations of Asn 74 to negatively charged residues lowered activities, while those to positively charged amino acids elevated activities in both the methyl green assay

(Fig. 5C) and the plasmid digestion assay (data not shown). Proline substitution reduced the activity in both assays by more than tenfold, perhaps due to adverse positioning of this loop region which contains the critical contact provided by Tyr 76 (Doherty et al., 1995). The enhanced activity in both assays associated with N74S may be explained by better hydrogen bonding capability of the serine hydroxyl group.

Mutational analysis of positions 14, 110, and 205 is also consistent with the trend of much lower activity for a negatively charged amino acid substitution and slightly higher activities for positively charged amino acid replacements (Fig. 5B,D). The methyl green activities for the other positive charge substitutions, including T10K, S75K, S75R, and T207K were lower than wild type by twofold to fourfold (Fig. 5D), perhaps due to DNA contacts provided by these wild-type residues or to the steric clashes with DNA introduced by the bulky lysine or arginine side chains. S75K also possessed a lower overall nicking activity but a higher linear to relaxed product ratio in the supercoiled plasmid digestion assay, suggesting tighter DNA binding and consistent with the 1.5-fold increase in the linear plasmid digestion activity of this variant (Pan & Lazarus, 1997). Finally, the removal of the wild type aspartic amino acid at position 107 lowered the methyl green activity by approximately twofold (Fig. 5D), presumably due to

the role of Asp 107 as a ligand for one of the structural calcium binding sites in DNase I (Oefner & Suck, 1986; Wolf et al., 1995). In conclusion, unlike the seven critical DNA-contacting residues that are close to the active site, residues that are peripheral to the active center play relatively minor roles in the activity of human DNase I.

Comparison of human vs. bovine pancreatic DNase I

Human DNase I shares 78% sequence identity with the bovine enzyme; the X-ray crystal structure, recently solved at 2.2 Å resolution, superimposes onto bovine DNase I (Weston et al., 1992) with an overall RMS deviation for main chain atoms of only 0.56 Å (Wolf et al., 1995). Of all the residues that directly contact DNA in either bovine DNase I-octamer complex (Lahm & Suck, 1991; Weston et al., 1992), only two are different in the human enzyme. At positions 9 and 206, human DNase I has a Gln and Pro, respectively, whereas Arg and Ser are found in bovine DNase I (Fig. 1). We have made the human variants Q9R and P206S in an attempt to explain the 2.4-fold higher activity of the bovine enzyme observed in the methyl green assay (Sinicropi et al., 1994). Compared to human wild-type DNase I, the methyl green activity of Q9R was 1.7-fold higher while that of P206S was approximately the same (Fig. 5A,D).

Consistent with earlier studies (Price, 1975), bovine pancreatic DNase I is most active when both Mg^{2+} and Ca^{2+} ions are included in the reaction mixture (Fig. 6A and Table 1). Its overall plasmid digestion activity, as measured by the rate of loss of the supercoiled substrate, is higher in Mn^{2+} alone than in Ca^{2+} or Mg^{2+} . Like its bovine counterpart, human DNase I also has maximal activity in the presence of Ca^{2+} and Mg^{2+} , and greater activity in Mn^{2+} alone than in Ca^{2+} or Mg^{2+} (Fig. 6B and Table 1). Unlike the bovine enzyme, the human version has a much higher activity in Mg^{2+} than in Ca^{2+} . The two enzymes also display

Table 1. Metal ion dependence of the overall nicking activities of bovine and human DNase I in the supercoiled plasmid digestion assay^a

Metal ions	Bovine DNase I	Human DNase I
None (EDTA)	0.005	0.01
Ca^{2+}	31	39
Mg^{2+}	47	780
Mn^{2+}	310	3,600
Ca^{2+} , Mg^{2+}	3,100	8,100
Ca^{2+} , Mn^{2+}	390	3,900

^aThe overall plasmid nicking activities are reported in $mg\ DNA \cdot min^{-1} \cdot mg^{-1}\ DNase\ I$.

different patterns of plasmid digestion in the presence of Ca^{2+} and Mg^{2+} , Mn^{2+} , or Ca^{2+} and Mn^{2+} . Bovine DNase I yields a significantly higher linear to relaxed product ratio than the human enzyme, suggesting that the former utilizes more double-stranded cutting than the latter. Finally, both nucleases exhibited only trace amounts of activity in the presence of 1 mM EDTA, which may account for the nick in the DNase I-Octa I co-crystal in the presence of 15 mM EDTA (Lahm & Suck, 1991).

Discussion

We have constructed over 80 human DNase I variants to address the significance of various residues at the DNA binding interface. The structural integrity of these variants remained intact based upon their ability to bind G-actin with the same high affinity ($\sim 1\ nM$) as wild type (Ulmer et al., 1996). In addition, all variants reacted with polyclonal anti-human DNase I antibodies, implying that they are properly folded. The results presented here, together with the structural information, allow us to propose the classification of these positions into four groups based on their different functional roles (Fig. 1). The first class is absolutely essential for cleavage of DNA and contains the two catalytic histidines, 134 and 252, along with their respective hydrogen bond partners Glu 78 and Asp 212. The second class is made up of the three crucial acidic residues—Glu 39, Asp 168, and Asp 251—that may serve as ligands for metal ion chelation. The third class consists of seven critical DNA-contacting positions—Gln 9, Arg 41, Tyr 76, Arg 111, Asn 170, Tyr 175, and Tyr 211—that encircle the active site. Finally, the fourth class includes 13 residues—Thr 10, Glu 13, Thr 14, Ser 43, His 44, Arg 73, Asn 74, Ser 75, Asp 107, Asn 110, Thr 205, Pro 206, and Thr 207—that interact with DNA distal from the active site and contribute much less to the activity of DNase I. Therefore, although as many as 15 direct DNA contacting residues have been detected in the two crystal structures of DNA-DNase I complexes (Lahm & Suck, 1991; Weston et al., 1992), only those seven contacts that are immediately adjacent to the scissile bond are critical for activity. The other contacts may contribute to the general stability of the protein-DNA complex as a group interacting with more peripheral nucleotides and thus could tolerate more variations. Not surprisingly, a sequence comparison of the catalytic, metal-ion coordinating, and DNA recognition positions show that these positions from the first three classes are more conserved among DNase I-like proteins across different species than those of the fourth class (Table 2).

Fig. 6. Plasmid DNA digestion by bovine (A) and human (B) pancreatic DNase I. Supercoiled pBR322 substrate with a low level of relaxed circle background (first lane, "C" for control) was digested with different amounts of DNase I for 42 min in the presence of EDTA or 1, 3, and 10 min in the presence of various metal ions. The linear product (L), relaxed product (R), and supercoiled substrate (S) are denoted.

Our results with human pancreatic DNase I are generally consistent with those found with the bovine enzyme by Connolly and coworkers. Worrall and Connolly demonstrated the importance of positions 39 and 134 by estimating the relative activities of Q38E:E39Q and H134Q mutants for cleaving the 160 bp tyr T promoter DNA at ~ 0.01 and $\sim 0.001\%$, respectively, of the native enzyme (Worrall & Connolly, 1990). Doherty and coworkers made mutations at the DNA binding positions Arg 41 and Tyr 76; in particular, they detected only 0.24% and 0.48% of the wild-type activity for the R41A and Y76A mutants, respectively, by using the DNA hyperchromicity assay (Doherty et al., 1991; Doherty et al., 1995). More recently, additional active site mutations including R9A, E78T, D168S, D212S, and H252Q have been made (Jones et al., 1996). The R9A and E78T bovine variants have $\sim 10\%$ and $\sim 1\%$ of the wild-type activity, respectively, while the other three possess virtually no detectable activity.

Human vs. bovine DNase I

The conservation of most of the DNA recognition and catalytic residues between the human and bovine DNase I (Table 2) may explain their same requirement for Mg^{2+} and Ca^{2+} for maximal activity (Table 1). However, there are some significant differences between them. For instance, in the presence of Mg^{2+} and Ca^{2+} ions human DNase I is ~ 2.4 -fold less active than the bovine enzyme in the DNA-methyl green assay (Sinicropi et al., 1994), but ~ 2.6 -fold more active in the supercoiled plasmid digestion assay (Table 1). This apparent inconsistency between the two assays can be explained by the greater linear to relaxed product ratio for the bovine nuclease (Fig. 6). This phenotype has previously been observed for the hyperactive human DNase I variant E13R:N74K (Pan & Lazarus, 1997). The methyl green assay may be measuring both double-stranded breakage and single-stranded nicks, while the overall nicking activity in the supercoiled plasmid digestion assay only depends on the latter.

The alignment of the human and bovine DNase I sequences reveals only two positions (9 and 206) that are different out of the residues involved in catalysis, direct DNA contact, and metal ion chelation (Lahm & Suck, 1991; Weston et al., 1992). Replacing the human residues with those from the bovine sequence demonstrated that position 9, one of the eight hyperactive human DNase I positions that we identified previously (Pan & Lazarus, 1997), was the key difference between the two enzymes. The positively charged arginine of the hyperactive variant allowed DNase I to interact more tightly with the negatively charged DNA phosphate backbone, which led to a 2.3-fold higher linear to relaxed product ratio in the supercoiled plasmid digestion assay as well as a 2.4-fold increase in V_{max} in the DNA hyperchromicity assay (Pan & Lazarus, 1997).

A family of DNase I-like proteins

The overall amino acid sequence identities among DNase I from different species range from 75–93%. It is evident that the residues in the first three classes addressed in this study are quite important for the functional activity of DNA cleavage. With the exception of position 9 (Table 2), they are completely conserved among human (Shak et al., 1990), bovine (Lahm & Suck, 1991), murine (Peitsch et al., 1995), rat (Polzer & Mannherz, 1990), porcine (Paudel & Liao, 1986), and ovine (Paudel & Liao, 1986) DNase I. Except for residues 9, 41, and 175, all residues of the first three classes are again found in four human DNase I-like proteins, which include LS-DNase¹, DHP-1², DNase γ ², and DNLI¹ (Parrish et al., 1995;

Pergolizzi et al., 1996). The overall identities among the four human DNase I-like proteins and DNase I from the six different species generally range from 38–53%; one exception is LS-DNase and DNase γ which are 83% identical. The divergence observed at positions 9, 41, and 175 is consistent with our alanine mutagenesis data at these positions (Fig. 2), showing that they are more tolerant to substitution than other members of the first three classes.

Recently, a three-dimensional structure of bacterial sphingomyelinase (SMase), a Mg^{2+} -dependent cholinephosphohydrolase, has been proposed using a protein fold recognition method (Matsuo et al., 1996). The predicted structure of SMase is highly compatible with the bovine DNase I structure (Oefner & Suck, 1986), even though the total sequence identity is less than 10%. Despite the lack of overall sequence similarity between mammalian DNase I and bacterial SMases, six out of the seven positions in the catalytic and metal-ion binding classes are conserved, and the homologous aspartic acid substitution is found at position 78 for two of the SMases (Table 2). Furthermore, positions 111 and 170, which interact with either side of the scissile bond of DNA and are the most important members of class three based on our alanine mutagenesis data (Fig. 2B), are also conserved between the two types of enzymes. Thus, these two classes of phosphodiesterases may have evolved from a common ancestor employing the same residues for catalysis. Deoxyribonucleases evolved the ability to recognize DNA by gaining additional binding residues of the third class. Native human DNase I displays poor affinity to DNA based upon surface plasmon resonance and gel-retardation studies (data not shown). The low substrate affinity could explain the dramatic reduction in activity when any one of the critical contacts in class three was removed (Fig. 2B).

Although the native residues in the fourth class appear to contribute much less to the activity of DNase I, introducing positively charged amino acids at these peripheral positions (e.g., N74K, N110R) results in a substantial increase in activity and a change in mechanism from nicking to processive cutting (Pan & Lazarus, 1997, 1998). Therefore, the evolutionary choice of the residues found at these positions is noteworthy since relatively minor mutations can have such significant effects.

Materials and methods

Mutagenesis, expression, and characterization

Site directed mutagenesis, expression in human 293 cell culture, human DNase I ELISA, and actin binding ELISA measurements were carried out essentially as previously described (Ulmer et al., 1996). The cell culture fluid from the DNase I-transfected 293 cells was collected and concentrated 10-fold; the resulting DNase I variant concentration was generally $>10 \mu\text{g/mL}$. The actin binding affinity of all of the variants described herein was the same as wild type.

Supercoiled plasmid digestion assay

Supercoiled pBR322 plasmid DNA (New England Biolabs) at $30 \mu\text{g/mL}$ was incubated with purified bovine DNase I (Worthington Biochemicals) or diluted culture media from human DNase I-transfected 293 cells in the presence of 25 mM Hepes, pH 7.0, 100 $\mu\text{g/mL}$ bovine serum albumin, and 4 mM $MgCl_2$, $MnCl_2$, and/or $CaCl_2$. For conditions containing only Mg^{2+} or Mn^{2+} ions, 0.4 mM EGTA was also included in the reaction mix to sequester trace Ca^{2+} ions present in solution. At various time intervals,

aliquots of the reaction mix were quenched with 10 mM EDTA, 10 mM EGTA, 6% glycerol, xylene cyanol and bromophenol blue and loaded directly on a 0.8% agarose gel. For reactions in the absence of metal ions, DNase I was mixed with the plasmid DNA in the presence of 1 mM EDTA for 42 min prior to quenching, followed by immediate loading on the 0.8% agarose gel. Gels were run overnight at ~1 V/cm in TBE (90 mM Tris-borate and 2 mM EDTA), stained with ethidium bromide, and individual bands were quantified with a Molecular Dynamics Model 575 FluorImager. Overall plasmid nicking activity was measured as the rate of disappearance of supercoiled plasmid DNA which was linear over the initial time points. The linear to relaxed product ratio was also determined from the initial rates of appearance of linear and relaxed products. Mock-transfected media controls were run and, in general, yielded no significant background activities. Assays containing Mn^{2+} ions alone were used for the least active variants because of the higher linear to relaxed product ratios. Figures were prepared by using Adobe Photoshop 3.0.5.

DNA-methyl green assay

The methyl green assay was used to measure DNA hydrolytic activity of DNase I in the presence of Mg^{2+} and Ca^{2+} ions as described previously (Sinicropi et al., 1994). DNase I concentrations were determined by ELISA, using a goat anti-DNase I polyclonal antibody coat and detecting with a rabbit anti-DNase I polyclonal antibody conjugated to horseradish peroxidase. In both assays, multiple sample dilutions were compared to standard curves of wild-type DNase I to determine concentrations. Wild-type DNase I has a specific activity of 1300 MG-units/mg (Sinicropi et al., 1994); variants with less than ~10 MG-units/mg cannot be detected in this assay. The data are presented as percent activity of wild-type DNase I.

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