Site-Directed Mutagenesis of Phosphate-Contacting Amino Acids of Bovine Pancreatic Deoxyribonuclease I†

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Received October 19, 1998; Revised Manuscript Received January 19, 1999

ABSTRACT: Bovine pancreatic deoxyribonuclease I (DNase I) is an endonuclease which cleaves double-stranded DNA. Cocrystal structures of DNase I with oligonucleotides have revealed interactions between the side chains of several amino acids (N74, R111, N170, S206, T207, and Y211) and the DNA phosphates. The effects these interactions have on enzyme catalysis and DNA hydrolysis selectivity have been investigated by site-directed mutagenesis. Mutations to R111, N170, T207, and Y211 severely compromised activity toward both DNA and a small chromophoric substrate. A hydrogen bond between R111 (which interacts with the phosphate immediately 5′ to the cutting site) and the essential amino acid H134 is probably required to maintain this histidine in the correct orientation for efficient hydrolysis. Both T207 and Y211 bind to the phosphate immediately 3′ to the cleavage site. Additionally, T207 is involved in binding an essential, structural, calcium ion, and Y211 is the nearest neighbor to D212, a critical catalytic residue. N170 interacts with the scissile phosphate and appears to play a direct role in the catalytic mechanism. The mutation N74D, which interacts with a phosphate twice removed from the scissile group, strongly reduced DNA hydrolysis. However, a comparison of DNase I variants from several species suggests that certain amino acids, which allow interaction with phosphates (positively charged or hydrogen bonding), are tolerated. S206, which binds to a DNA phosphate two positions away from the cleavage site, appears to play a relatively unimportant role. None of the enzyme variants, including a triple mutation in which N74, R111, and Y211 were altered, affected DNA hydrolysis selectivity. This suggests that phosphate binding residues play no role in the selection of DNA substrates.

Bovine pancreatic deoxyribonuclease I (DNase I), a Ca^{2+}/Mg^{2+}-dependent double-stranded DNA endonuclease, has been the subject of extensive structural investigations by X-ray crystallography (*1*−*6*). These studies have shown that the protein binds to the minor groove of DNA, causing it to widen from 12 to 15 Å and bend in the direction of the major groove. Two amino acids, R41 and Y76, insert into the minor groove of DNA, with R41 hydrogen bonding to the bases and Y76 forming an unusual hydrophobic stacking interaction with one of the deoxyriboses. Some time ago we synthesized a gene coding for DNase I and expressed the protein in *Escherichia coli* (*7*), allowing us to investigate the roles of key amino acids using site-directed mutagenesis. Alterations to R41 and Y76 reduce the rate at which DNA is hydrolyzed but have little effect on DNA hydrolysis selectivity (*8*−*10*). DNase I cuts DNA with a low specificity, and poly(dA)-poly(dT) sequences, in particular, are poorly cut (*11*−*15*). Originally, this was attributed to the narrower than normal minor groove width of these sequences (*12*). However, R41 and Y76 are unlikely to be directly involved in "measuring" minor groove dimensions as double mutants with smaller side chains, e.g., R41A/Y76A, efficiently discriminate against poly(dA)-poly(dT) sequences. This argues against selectivity being a consequence of the fit of these amino acids into "typical" minor groove dimensions (*8*−*10*). More recent thinking explains the selectivity of DNase I in terms of DNA flexibility, more flexible sequences being better substrates (*16*−*18*). Thus the main role of R41 and Y76 is to couple the binding of DNA to its hydrolysis; insertion of these two amino acids into the minor groove giving rise to the DNA bending required to position the scissile phosphate at the active site.

Most of the contacts between DNase I and DNA do not involve interactions with the minor groove but, rather, the phosphate backbone. Several amino acids, including H134, D168, and H252, contact the scissile phosphate and play a direct role in the hydrolysis mechanism by acting as general acids/bases or coordinating essential metal ions (*2*, *6*). These three amino acids have been shown to be essential by site-directed mutagenesis (*19*). The remaining DNase I/DNA phosphate interactions may be divided into two groups: (1) contacts involving the peptide backbone and phosphates on the strand opposite that cut; (2) interactions between amino acid side chains and phosphates on the cleaved strand (*4*−*6*). Amino acids E13, T14, and S43 are involved in the peptide bond mediated contacts and N74, R111, N170, S206, T207, and Y211 in the side chain dependent interactions (Figure 1). N170 contacts the scissile phosphate while the remaining residues interact with phosphates flanking the cleavage site by one or two bases in either the 3′ or the 5′ direction. Recently (*20*), using human DNase I, it has been

† This work was supported by grants from the U.K. BBSRC and Wellcome Trust to B.A.C. S.J.E., E.J.S., and W.N.M. were/are BBSRC-supported Ph.D. students.

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10.1021/bi9824893 CCC: $18.00
Published on Web 03/09/1999

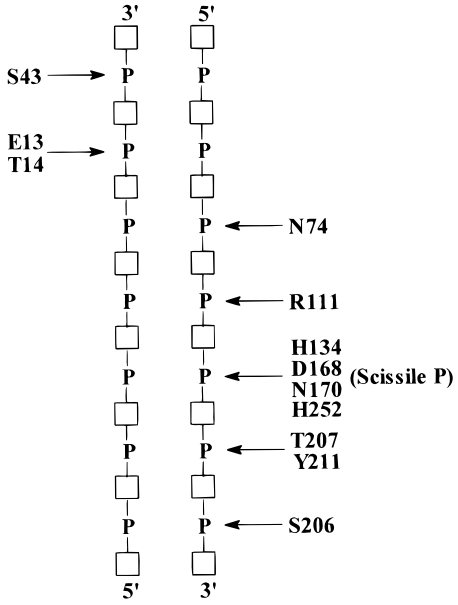

FIGURE 1: Schematic diagram of the interaction between DNase I and the DNA phosphates (adapted from ref *6*). The amino acids interacting with the phosphates in the left-hand strand do so via the peptide bond. In contrast, the amino acids that contact the phosphates in the right-hand strand use their side chains to make the interaction.

shown that converting N74 to a positively charged amino acid resulted in better DNA hydrolysis; in contrast, N74E was a much poorer enzyme. Generally, the introduction of positively charged amino acids, near the DNA, resulted in better catalysis.

The roles of the phosphate-contacting amino acids, in DNase I, have yet to be fully elucidated. Possible functions include the efficient binding of DNA and/or, as for R41 and Y76, the coupling of DNA recognition to effective hydrolysis. Although DNase I selectivity is currently largely explained in terms of DNA flexibility, mechanisms that rely on a sensing of the minor groove width cannot be fully discounted. It has been proposed that the extensive contacts to the phosphates, which take place at both strands of the helix, may provide a means for measuring the dimensions of the minor groove (*21*). To shed more light on both the hydrolysis and recognition of DNA by DNase I, this paper details mutagenesis experiments on the phosphate-contacting residues N74, R111, N170, S206, T207, and Y211. Their roles are examined by following the hydrolysis of calf thymus DNA and the small synthetic substrate thymidine 3′,5′-di-(*p*-nitrophenyl) phosphate. DNA hydrolysis selectivity has been examined using an *E. coli tyr*T promoter DNA fragment (*12*). This allows an evaluation of the influence that individual protein−DNA contacts have on substrate selectivity, especially on tracts of DNA normally particularly resistant or susceptible to hydrolysis.

MATERIALS AND METHODS

Materials. Bovine pancreatic DNase I was purchased from Worthington Biochemicals (Irvine, Scotland) and was of the highest purity available. This material was further purified by anion-exchange chromatography (*7*, *22*).

Site-Directed Mutagenesis of DNase I. A variety of methods were used to prepare DNase I mutants. (A) The *dut*$^-$ *ung*$^-$ method (*23*) with the following primers: N74D, 5′ GAACCCCTCGGGCGTGACTCTTACAAAGAACGGT 3′; R111K, 5′ AACGACTCTTTCTCCAAAGAACCGGCTGTTGTT 3′; R111A-5′ AACGACTCTTTCTCCGCTGAACCGGCTGTTGTT 3′; R111E-5′ AACGACTCTTTCTCCGAAGAACCGGCTGTTGTT 3′; S206A-5′ GACACCACCGCTACTGCTACCAACTGCGCTT 3′; Y211F-5′ AGTACCAACTGCGCTTTCGACCGTATCGTTGTT 3′; Y211A, 5′ AGTACCAACTGCGCTGCTGACCGTATCGTTGTT 3′. (B) "QuikChange" using a commercially available kit (Stratagene Ltd., Cambridge, U.K.) with the following primers: N170A, 5′ GCTGATGGGTGACTTCGCTGCTGACTGCTCTTATG 3′; T207A, 5′ GCTACTAGTGCCAACTGCGCT 3′; T207V, 5′ GCTACTAGTGTCAACTGCGCT 3′. (This method requires two primers, the second being complementary to those given.) (C) PCR mutagenesis (*24*) with the following primer: N170S, 5′ GGTTGACTTCTCTGCTGACTGC 3′.

In all cases the codon used to bring about the mutation is underlined. The *dut*$^-$ *ung*$^-$ mutagenesis was carried out using, as a template, a single-stranded M13mp19 derivative containing a gene coding for wild-type DNase I (*7*). The QuikChange and PCR mutageneses used double-stranded pAD10 (*9*, *22*) as templates. This is a pET11 (*25*) derivative, containing a gene coding for wild-type DNase I. The triple mutant, N74A-R111A-Y211A was prepared by the *dut*$^-$ *ung*$^-$ method starting from R111A. Two primers were used, in a simultaneous reaction, to introduce N74A (GAACCCCTCGGGCGTGCCTCTTACAAAGAACGGT) and Y211A (primer given above). In all cases the entire gene was sequenced both to confirm the presence of the mutation and to ensure no alterations at other sites.

Expression and Purification of Mutant Proteins. Most of the mutant proteins were expressed in derivatives of pAD10 (*9*, *22*). Protein expression was achieved either by induction with λCE6 phage (JM105) (N74D, R111K, R111A, R111E, S206A, Y211F, Y211A, triple mutant) or with IPTG (BL21 DE3) (T207V, T207A) as previously described (*9*, *22*). N170A and N170S were expressed in derivatives of pKK223-3 (JM105) (*7*, *8*, *19*). Mutant proteins were purified essentially according to published procedures (*7*, *22*) with the addition of a gel filtration step (*10*). Samples were concentrated using Centriprep-10 concentrators and stored as 50% glycerol stocks at −20 °C. The final purity of the DNase I mutants was checked by Coomassie blue stained SDS−polyacrylamide gels (*26*). Protein concentrations of mutant DNase I stock solutions were determined by absorbance at 280 nm using a molar extinction coefficient of 3.8×10^4 M^{-1} cm^{-1}.

Hydrolysis of DNA, Thymidine 3′,5′-Di(p-nitrophenyl) Phosphate, and E. coli tyrT Promoter DNA by DNase I Mutants. These assays were carried out as described (*9*). Steady-state kinetic data, obtained from the hydrolysis of DNA (Kunitz assays), were fitted to the Michaelis−Menton equation using GraFit (*27*).

RESULTS

Preparation and Purification of Phosphate-Contacting Mutants of DNase I. Mutations were made to amino acids N74, R111, N170, S206, T207, and Y211 as detailed in Table 1. The mutants were expressed using either derivatives of pAD10 (*9*, *22*) or pKK223-3 (*7*, *8*, *19*). Plasmid pAD10 is derived from the T7 RNA polymerase based expression

Table 1: Kinetic Properties of the DNase I Mutants Investigated in This Study[a]

DNase I variant	K_m (mg/mL) (Kunitz assay)	V_{max} (Kunitz units) (Kunitz assay)	V_{max}/K_m (rel) (Kunitz assay)	activity (rel) toward thymidine 3′,5′-di(*p*-nitrophenyl) phosphate
wild type	0.03 ± 0.005	$(8.6 \pm 0.3) \times 10^5$	100	100
N74D	0.07 ± 0.02	$(1.9 \pm 0.3) \times 10^3$	0.09	31
R111K	0.03 ± 0.01	$(1.2 \pm 0.3) \times 10^4$	1.4	0
R111A		0	0	0
R111E		0	0	0
N170A	not determined	not determined	0.005[b]	3
N170S	not determined	not determined	0.008[b]	2
S206A	0.02 ± 0.005	$(1.8 \pm 0.2) \times 10^4$	3	49
T207A		0	0	0
T207V		0	0	0
Y211F	0.04 ± 0.01	$(1.8 \pm 0.2) \times 10^2$	0.016	90
Y211A		0	0	0
N74A-R111A-Y211A		0	0	0

[a] The V_{max}/K_m values for the Kunitz assay are expressed relative to the wild-type enzyme (=100). The activities toward thymidine 3′,5′-di(*p*-nitrophenyl) phosphate are also expressed relative to the wild-type enzyme (=100). As mentioned in the text, the results at several concentrations of this synthetic substrate were averaged to produce the values given. [b] Activity ratio (mutant rate/wild-type rate) at a single DNA concentration of 0.05 mg/mL as rates were too slow to determine kinetic parameters. The K_m and V_{max} values were determined at least three times.

vector pET11 and, when used with λCE6 bacteriophage to introduce the T7 RNA polymerase, allows an extremely high level of transcriptional control (*25*). This prevents "leaky" expression of potentially toxic products and has been very useful for the production of wild-type and highly active variants of DNase I (*9*, *22*). Alternatively, an *E. coli* strain (BL21 DE3), which has the *T7 polymerase* gene under *lac* operator control, allowing slightly less tight control of gene expression, can be used. Most of the mutants have been expressed using the pAD10 system. Although we originally developed this system for active DNase I, it also gives very good results with low-activity mutants. N170A and N170S were expressed in derivatives of pKK223-3, which is also suitable for less active DNase I variants (*7*, *8*, *19*). Each of the mutant proteins, which are listed in Table 1, was purified using a three-column procedure based on DEAE-Sephacel followed by FPLC on a DEAE column and finally gel filtration (*7*, *8*, *22*). The purity of the mutants, as assessed by SDS−PAGE using Coomassie blue stain, was greater than 97% (not shown). The activity of the mutants was compared to that of purified bovine pancreatic DNase I, which has properties identical to those of the recombinant wild-type enzyme (*7*).

Hydrolysis of DNA by DNase I Mutants. The interaction of the DNase I mutants with DNA has been determined using the Kunitz assay, which measures the hydrolysis of calf thymus DNA using the hyperchromic effect (the increase in absorbance, at 260 nm, as double-stranded DNA is degraded to single strands) (*7*, *28*−*30*). The digestion of calf thymus DNA by DNase I has been shown to obey Michaelis−Menten kinetics providing DNA concentrations below 0.1 mg/mL are used. Higher substrate concentrations are inhibitory (*7*, *28*, *31*). However, due to the undefined nature of calf thymus DNA, it is difficult to convert K_m and V_{max} values to commonly used units (*22*). Previously, mg/mL DNA (rather than nM) has been used for K_m and Kunitz units [rather than nmol of product produced min^{-1} (mg of enzyme)$^{-1}$] for V_{max} (1 Kunitz unit is defined as an increase in absorbance of 0.001 min^{-1} (mg of enzyme)$^{-1}$. Nevertheless, these apparent K_m and V_{max} values have been very useful for comparing mutants of DNase I with each other and the wild-type enzyme (*19*, *20*, *22*).

The kinetic parameters obtained for the hydrolysis of DNA by wild-type DNase I and active mutants were obtained by direct fits to the Michaelis−Menton equation using GraFit (*27*). Representative examples are given in Figure 2, and the results are summarized in Table 1. No activity was detected for R111A, R111E, T207A, T207V, and Y211A. Thus, these five mutants were judged to have residual activities of the same order as the active site mutants H134Q and H252Q. Both of these mutants were 10^5-10^6 times less active than wild-type DNase I in Kunitz assays (*19*). Altering N170 to A or S resulted in just measurable activities of 0.005% and 0.008%, respectively. However, these levels of activity are too low to allow K_m and V_{max} determination, and thus catalysis was measured at the single DNA concentration of 0.05 mg/mL. Y211F had a low activity, with the V_{max}/K_m reduced to 0.016% of the wild type. Most of the reduction resulted from a drop in V_{max}. R111K had a V_{max}/K_m 1.4% of wild type entirely due to a drop in V_{max}. N74D also showed a low V_{max}/K_m. The slight increase in K_m may be due to electrostatic repulsion between the phosphate and carboxylate side chain, but once again a decrease in V_{max} predominates. Amino acids N74, R111, N170, T207, and Y211 appear to play an important role in DNA hydrolysis, at least as assessed by the mutations we have used, all of which are poorly tolerated. Only for S206A is the reduction in V_{max}/K_m, to 3% of wild type, not too severe, and this amino acid seems less critical for DNA hydrolysis. As expected from the results of the single mutants, the triple mutant N74A-R111A-Y211A showed no activity.

Hydrolysis of Thymidine 3′,5′-Di(p-nitrophenyl) Phosphate by DNase I Mutants. DNase I binds to this substrate very weakly, and a different bond is cleaved compared to that in DNA (*32*). It is unlikely that most of the interactions seen between DNase I and DNA, which involve contacts in the minor groove and to phosphates, will occur with this small substrate, explaining its poor binding. Nevertheless, this small chromophore binds to the same active site as DNA, and the same catalytic residues are used for the hydrolysis of both substrates (*9*, *19*). Thus, thymidine 3′,5′-di(*p*-nitrophenyl) phosphate hydrolysis may be used to examine the effects that mutagenesis to residues, remote from the active site, have on enzyme activity due to gross conformational changes

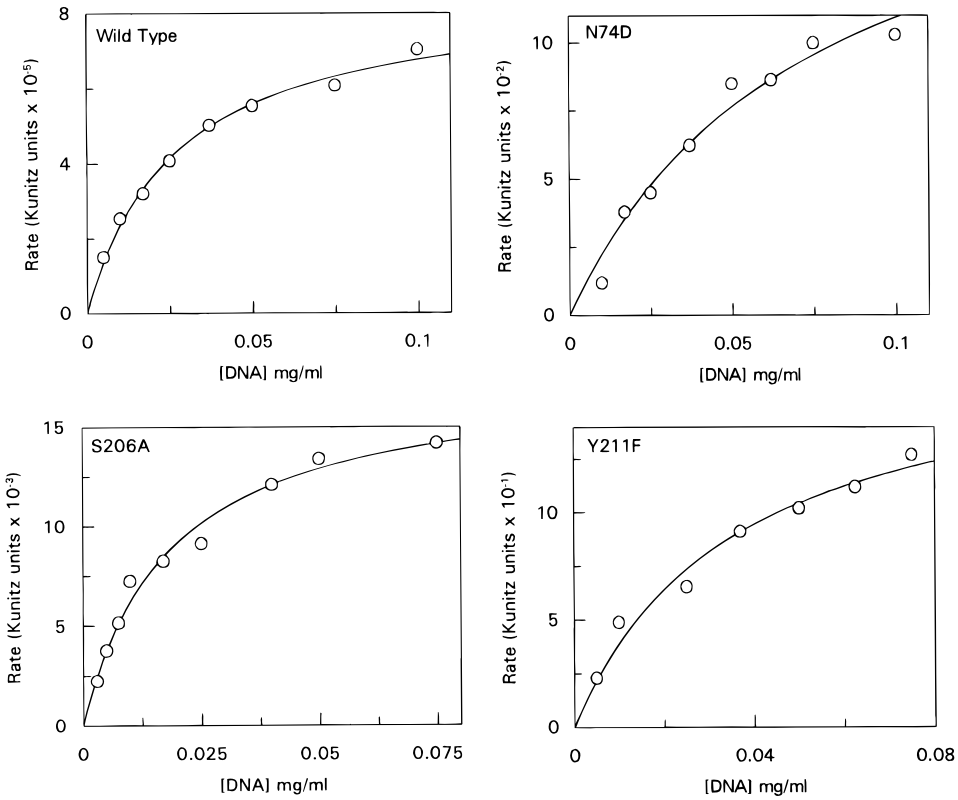

FIGURE 2: Hydrolysis of DNA by wild-type DNase I and the mutants N74D, S206A, and Y211F using the Kunitz assay. The lines shown are fits to the Michaelis−Menton equation using GraFit (*27*).

or small alterations to the positions of key catalytic amino acids (*9*).

The cleavage of thymidine 3′,5′-di(*p*-nitrophenyl) phosphate by DNase I does not obey Michaelis−Menton kinetics, compromising the measurement of K_m and V_{max} values (*31*, *32*). More than one molecule of this substrate appears to bind to DNase I in a cooperative manner, most probably stacking on top of each other and filling the DNA binding site (*32*, *33*). Previously, we determined the activity of several DNase I mutants at various concentrations of this synthetic substrate. It was observed that the ratio (activity mutant)/(activity wild type) was invariant with thymidine 3′,5′-di(*p*-nitrophenyl) phosphate concentration. This allowed the average of the ratio seen at the different substrate concentrations to be used as a measure of activity of each mutant (*9*, *19*). Therefore, the activities of the DNase I variants, used in this study, were evaluated at several thymidine 3′,5′-di(*p*-nitrophenyl) phosphate concentrations between 2 and 15 mM. The ratio (activity mutant)/(activity wild type) was determined at each concentration (as with previous mutants, this was found not to vary), and the average of this ratio, for each mutant, is given in Table 1. No activity was detected for the three R111 and the pair of T207 mutants. These two amino acids are required for the efficient hydrolysis of both DNA and chromophoric substrates and thus behave like amino acids H134, D168, and H252, for which direct roles in the catalytic mechanism have been proposed (*6*, *19*). The two N170 mutants showed measurable activity with thymidine 3′,5′-di(*p*-nitrophenyl) phosphate, about 2−3% of the wild-type rate. Both N74D and S206A retain reasonably high activity (31% and 49%, respectively) and therefore can be discounted as major contributors to catalysis. The most interesting result was obtained with Y211, with Y211A being completely inactive coupled with high retention of activity (90%) by Y211F. It is most likely that this amino acid is critical for enzyme activity and that the conservative change Y211F is tolerated. Removal of the aromatic ring, in the mutant Y211A, probably perturbs the active site by causing conformational changes that lead to the movement of critical catalytic residues. The triple mutant N74A-R111A-Y211A showed no activity.

Sequence Selectivity of DNase I Mutants. The limited digestion of *E. coli tyr*T promoter DNA by DNase I produces a characteristic cleavage pattern when analyzed by gel electrophoresis (*12*). This substrate is useful for investigating the sequence selectivity of DNase I since it contains regions or "gaps" which exhibit a higher than average resistance to cleavage. Gaps 1 and 2 are A/T rich and are probably hydrolysis resistant due to their limited flexibility (*9*, *34*). Gap 3 is G/C rich, and it was originally proposed that such regions were also DNase I resistant (*12*). However, cutting in this region does not exhibit such a marked resistance to cleavage as A/T-rich areas. In addition, neighboring phosphates are often cut at very different rates, and thus this DNA fragment can be used to study "local" sequence effects (*12*). The *tyr*T fragment has previously been used to examine the cleavage characteristics of DNase I mutants that have alterations to minor groove binding amino acids (*8*−*10*) and active site mutants (*19*).

Digestion of both the 3′ → 5′ and 5′ → 3′ strands of the *tyr*T DNA by all the phosphate-contacting mutants produced cleavage patterns that were essentially the same as that seen for wild-type DNase I. This was true for all the single amino acid mutants and even the triple mutant N74A-R111A-Y211A. As an example, the hydrolysis ladders found, for the 3′ → 5′, with R111K, R111A, and R111E are shown in

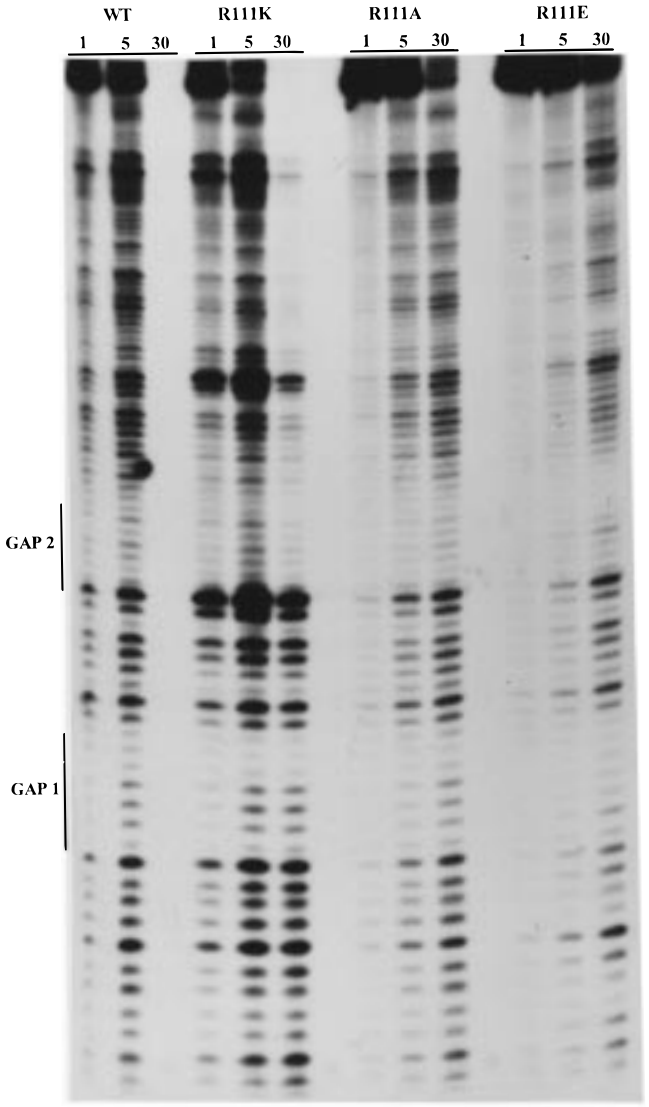

FIGURE 3: Results of digesting the 3′ → 5′ strand of *tyr*T promoter DNA with wild-type DNase I and the mutants R111K, R111A, and R111E. The times of digestion (minutes) are shown on the top of each track. Gaps 1 and 2 (illustrated) are A/T-rich regions which are slowly hydrolyzed by wild-type DNase I (*12*). A full sequence of the DNA used has been given previously (*9*).

Figure 3. Previous mutations, to the minor groove binding and active site amino acids, also gave hydrolysis patterns similar to those of wild-type DNase I. However, in most of these cases some minor differences were apparent. With the phosphate-contacting amino acids even small differences are hard to observe. The obvious conclusion is that none of the residues under current investigation play a role in determining the cleavage selectivity of DNase I. It should be noted that *tyr*T DNA hydrolysis, followed by gel electrophoresis, is an extremely sensitive assay. Therefore, mutants that give no apparent activity in Kunitz assays usually show activity in *tyr*T DNA cleavage.

DISCUSSION

This work addresses the roles of amino acids N74, R111, N170, Y211, S206, and T207 in DNA recognition and hydrolysis by DNase I. As shown in Figure 1, these amino acids make contacts to the scissile phosphate and to the four immediately flanking phosphates (two in the 3′ and two in the 5′ direction). The importance of individual amino acids in DNase I may be initially assessed by comparing the amino acid sequences from several species as shown in Table 2. R111, N170, and Y211 are absolutely conserved, suggesting that these amino acids may play a critical role. T207 is conserved, except in the pig enzyme where it is replaced by H. However, this assignment is based on amino acid, rather than DNA, sequencing (*35*), and this technique is not 100% reliable. The sequence reported for pig DNase I at amino acids 206, 207, and 208 is SHT. Gene sequencing gives STH for mouse (*36*) and rat (*37*), and with human (*38*) PTH is seen. We, therefore, believe that the positions of this T and H in the pig enzyme have been reversed, and the true sequence should be STH. If this is true, T207 is also absolutely conserved. N74 and S206 show some variability. N74 can be K, R, or S, and S206 is replaced by P and Q in some cases (Figure 2). It is interesting that the highly conserved R111, N170, T207, and Y211 make contacts at or immediately flanking the scissile phosphate. In contrast, the more variable N74 and S206 interact with phosphates two positions away from the cutting site. The two multifunctional repair enzymes exonuclease III (*39*) and human apurinic/apyrimidinic endonuclease (HAP1) (*40*) have considerable structural similarity to DNase I. Both exonuclease III and HAPI contain an asparagine and arginine that correspond to N170 and R111, respectively, in DNase I (*40*).

Table 2: Identity of the DNase I Amino Acids, Investigated in This Study, in Various Species

	amino acid					
species[a]	74	111	170	206	207	211
cow	N	R	N	S	T	Y
human	N	R	N	P	T	Y
rat	K	R	N	S	T	Y
mouse	K	R	N	S	T	Y
rabbit	R	R	N	S	T	Y
sheep[b]	N	R	N	S	T	Y
pig[b]	S	R	N	S	H	Y
fish (*Tilapia*)	S	R	N	Q	T	Y

[a] Sources: cow (*51*); human (*38*); rat (*37*); mouse (*36*); rabbit (*52*); sheep (*53*); pig (*35*); fish (*54*). [b] Determined by amino acid sequencing. All others determined by DNA sequencing.

The kinetic data (Table 1) obtained for mutations to R111, T207, and Y211 are in good agreement with the amino acid sequence data. In general, alterations to these amino acids either completely abolish or very strongly reduce activity toward both DNA and thymidine 3′,5′-di(*p*-nitrophenyl) phosphate. The only exception is the efficient hydrolysis of the synthetic substrate by Y211F. Amino acids R111, T207, and Y211 are in proximity of the active site, but none are near enough to the scissile phosphate to have a direct role in the catalytic mechanism (*5*, *6*). Why then does their alteration have such a pronounced affect on the rate of phosphodiester bond hydrolysis? In the case of DNA substrates we would like to propose the involvement of "networks" that link the phosphates, contacted by R111, T207, and Y211, to amino acids that play a key role in the catalytic mechanism. As shown in Figure 4, R111 not only makes a salt bridge to its target phosphate but also interacts with the peptide bond carbonyl oxygen of H134 (*1*). Y211, as well as interacting with a phosphate, is the nearest neighbor of D212. This aspartate is hydrogen bonded to

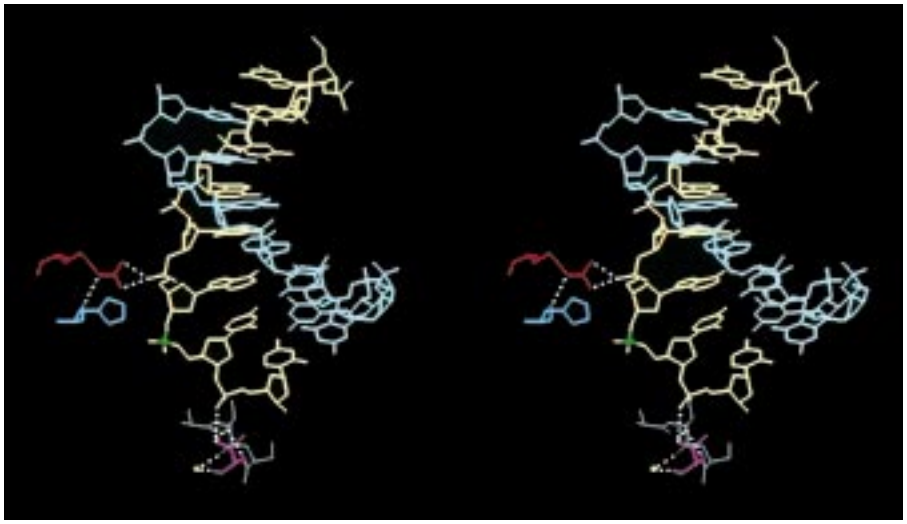

FIGURE 4: Interaction of the DNase I amino acids R111 (red) and T207 (magenta) with the two phosphates that flank the scissile phosphate (green). R111 also forms a hydrogen bond with the peptide backbone of H134 (dark blue), an amino acid near the scissile phosphate that plays an important role in the hydrolysis mechanism (*19*). T207 additionally interacts with a structural Ca^{2+} ion (yellow sphere) that helps to stabilize a threonine-rich loop (part of which is shown in gray). This loop maintains the integrity of an essential disulfide bridge between C173 and C209. Note the yellow sphere is actually a water molecule, rather than Ca^{2+}, in this protein−DNA complex, which was grown in the presence of EDTA to prevent hydrolysis. However, a Ca^{2+} ion occupies this position in free protein complexes, and in the full protein−DNA−metal ion complex it is also thought that Ca^{2+} is found at this site. The coordinates used to generate this side-by-side stereo picture are taken from a DNase I−d(GGTATACC)$_2$ complex (*6*). The hatched white lines represent hydrogen bonds.

H252. The two histidines, H134 and H252, are among the most important catalytic residues in DNase I. They flank the scissile phosphate and provide general acid/base catalysis, although it is still not certain which histidine acts as general acid and which as general base (*2*, *5*, *6*, *19*). Mutation to both of these histidines, and also D212, results in a severe drop in activity (*19*). It is easy to imagine that the interaction between R111 and H134 is critical for the correct positioning of the catalytically essential histidine. Similarly, Y211 may be important for the orientation of D212 and ultimately the alignment of H252. T207 is part of a threonine-rich loop that forms the binding site for one of the structural Ca^{2+} ions (*1*), and its backbone carbonyl oxygen is one of the metal ion ligands (Figure 4). The conformation of this loop, in the presence of Ca^{2+}, is important for the maintenance of the neighboring essential disulfide bridge, C173−C209 (*41*). Alterations to T207 may perturb Ca^{2+} binding and the critical disulfide and so lead to incorrect assembly of the active site. The results with thymidine 3′,5′-di(*p*-nitrophenyl) phosphate are more difficult to explain, as no structural data exist for a binary complex with DNase I. DNase I cuts this substrate, at its 3′ phosphate, with the release of nitrophenol (*32*). If the 3′-phosphate is located at the active site, at the same position as the scissile phosphate in DNA, then it is possible to place the 5′-phosphate in a suitable location to interact with R111. The effects that mutations to R111 have on thymidine 3′,5′-di(*p*-nitrophenyl) phosphate hydrolysis can then be explained in exactly the same way as for DNA. This binding orientation does not provide a phosphate for interaction with Y211, and a definitive explanation of the results seen when Y211 is altered cannot, at present, be given. A possibility is that wild-type DNase I and Y211F use a new hydrophobic interaction between the aromatic rings of the amino acid and the 3′-nitrophenol group to compensate for the loss of the hydrogen bond to the phosphate, giving catalysis. This interaction cannot occur with Y211A, resulting in the nonsubstrate seen.

The concept of "networks" linking the binding of DNA, both through bases and phosphates, to its hydrolysis is not new. A similar idea has been invoked to explain how the restriction endonuclease *Eco*RV responds to phosphorothioate substitutions (*42*). Indeed tightly coupled networks of interactions seem to be very common for DNA binding enzymes (*43*). Mutations to the DNase I amino acids Y76 and R41, which interact with a sugar and a base, respectively, in the minor groove of DNA, also greatly reduce the activity of the enzyme (*8*, *9*). Here, it was also suggested that the function of these two amino acids was to couple the binding of DNA to its hydrolysis. R111 and Y211 appear to play an identical role. Thus many amino acids in DNase I, which are remote from the active site and bind to the DNA via sugars, bases, or phosphates, behave in a similar manner. Their purpose is to ensure that binding of DNA leads to efficient catalysis, by correct juxtaposition of the scissile phosphate and the enzyme's catalytic machinery. DNase I is a very rigid enzyme that undergoes very little conformational change on binding to DNA. Thus the protein−protein components of the network are present in both the free enzyme and the enzyme−DNA complex. Following DNA binding, the protein−DNA contacts that are formed serve to distort the DNA, rather than the protein, and allow efficient catalysis to take place.

The clustering of R111, Y211, and T207 in close proximity to the catalytic core of DNase I is somewhat similar to that for RNase A from bovine pancreas, where noncatalytic subsites are situated adjacent to catalytic residues (*44*). Such noncatalytic residues have been shown to drastically affect the rate of cleavage of substrate although they do not contribute to the catalytic mechanism. Site-directed mutagenesis of phosphate-contact residues within these subsites to remove their positive charges (K7Q, R10Q) produces enzymes with greatly reduced activities, despite mutated residues not being directly involved in the catalytic mechanism of enzyme action (*45*). No function other than substrate

contact has been pinpointed for these residues; thus the possibility exists that such subsites, like the phosphate-contact residues of DNase I, are required to maintain the substrate in its correct conformation for optimal phosphodiester bond cleavage.

Unlike R111, T207, and Y211, N170 interacts directly with the scissile phosphate; a hydrogen bond is formed between its amide side chain and one of the nonesterified phosphate oxygens. Amino acids with an amide side chain have often been observed at the active site of nucleases, e.g., RNase A (*46*, *47*), *Serratia* nuclease (*48*), and the homing endonuclease I-*Ppo*I (*49*). In the case of the *Serratia* enzyme the importance of this amino acid has been confirmed by mutagenesis (*48*). However, with RNase A alterations of the active site glutamine did not markedly reduce activity (*50*). Roles suggested for these active site amides include binding of the scissile phosphate in the ground state, transition state stabilization, metal ion binding, and coordination of the hydrolytic water molecule, and therefore, this amino acid may play a direct role in catalysis. Mutations to N170 largely abolish the hydrolysis of both DNA and small substrates, although the reductions are less severe than for the other catalytic residues, H134, D168, and H252 (*19*). The simplest explanation is that this amino acid plays a direct role in the catalytic mechanism, a hypothesis supported by its conservation in DNase I from various species and the presence of an analogous amino acid in exonuclease III and HAP1. However, the exact mechanistic function of N170 awaits elucidation.

The amino acid N74 residue forms part of an important, surface, flexible loop (R73-N74-S75-Y76-K77-E78) centered on Y76. This loop inserts into the minor groove of DNA and widens it by 3 Å, with Y76 forming a hydrophobic stacking interaction with a sugar residue (*4*−*6*). It is likely that the function of N74 is to contact the relevant phosphate group and, in conjunction with Y76, set up the loop such that it may insert productively into the minor groove. We decided to study the effects of completely disrupting the N74−phosphate hydrogen bond with the mutation N74D. It is most unlikely that any interaction will form between this variant and the phosphate due to charge−charge repulsion. N74D cut DNA poorly, probably because of incorrect interaction of the loop with the minor groove of DNA and disruption to the Y76−sugar interaction. Y76 interacts with the key catalytic residue H134 not directly but through E78 (*9*). Thus, in a manner similar to that above, N74 is networked with H134, and changes to N74 therefore affect H134. In contrast, N74D cuts thymidine 3′,5′-di(*p*-nitrophenyl) phosphate efficiently. Similarly, mutations to Y76, or deletion of the entire loop, did not severely compromise the hydrolysis of the synthetic substrate (*9*). The flexible loop binds to the minor groove of DNA, an area that has no counterpart in thymidine 3′,5′-di(*p*-nitrophenyl) phosphate. Thus it is perhaps not surprising that changes to amino acids within the loop have little effect on the cleavage of the small substrate.

We considered making more conservative changes than N74D. However, such alterations already exist in nature. As shown in Table 2, alterations to N74 yielding either positively charged or hydrogen-bonding amino acids appear to be tolerated. A compilation of sequences of AP endonucleases, including exonuclease III and HAP 1 (*40*), shows that an amino acid of this type is usually present at this location. Unlike the aspartic acid, present in the mutant of N74D, the naturally occurring variants should still be able to interact with phosphate groups via either a salt bridge or a hydrogen bond. Our view is that the amino acid at position 74 again serves to couple DNA binding to hydrolysis. This amino acid is often N, but other amino acids, capable of binding phosphates, are tolerated. Support comes from demonstration that the mutants N74K and N74R, of human DNase I, have high activity, whereas N74E is poorly active (*20*).

Although S206 interacts with a phosphate, as do all the other amino acids studied here, its alteration has a more modest effect on DNA thymidine 3′,5′-di(*p*-nitrophenyl) phosphate hydrolysis than most of the other mutations. Certainly it appears to be the least important amino acid studied. This amino acid does not take part in the networks described above, and the drop in V_{max}/K_m seen with DNA may be due to the simple deletion of the interaction. Similarly, this amino acid is likely to be too far away from the active site to interact with the chromophoric substrate. The P found at position 206 in the human enzyme and Q for the fish emphasize the relative unimportance of this amino acid.

None of the mutations had any effect on the DNase I hydrolysis selectivity, as measured by the hydrolysis of *tyr*T promoter DNA. This includes alterations to N74, which is part of the loop that binds to DNA via the minor groove, and a triple mutant, N74A-R111A-Y211A, in which three of the phosphate-contacting groups are removed. Previously it was shown that changes to Y76 and deletion of the entire loop had very little effect on hydrolysis selectivity (*9*). Hence, the result with N74 is unsurprising. We have proposed that mutations to N74, R111, and Y211 may alter the disposition of active site amino acids. However, mutations to these amino acids rarely change selectivity (*19*), and so networking effects would not, per se, result in an alteration of specificity. The unchanging digestion patterns produced by phosphate-contacting DNase I mutants, compared to the wild-type enzyme, clearly indicate that they do not play any role in determining the specificity of DNA cleavage. Also, the idea that DNase I "senses" minor groove widths by measuring the distances between phosphates on opposing strands must be questioned. It seems reasonable to assume that the accurate recognition of precise minor groove width, by this mechanism, would necessitate a full complement of the amino acids responsible. In particular, the unchanging selectivity with the triple mutant would appear to invalidate this idea. All the mutations we have made to DNase I, including those to amino acids that interact with sugars, bases, and phosphates, as well as catalytically essential residues have had minor effects on DNA hydrolysis selectivity (*8*−*10*, *19*). Certainly DNA sequence variation leads to much more profound changes in selectivity (*11*−*13*) than do alterations to DNase I. We conclude that the determinants of DNA cleavage selectivity in the DNase I/DNA interaction lie with the nucleic acid rather than the protein. DNA sequences that are easy to flex and bend are well cut by DNase I. Therefore, unless mutants of DNase I can be found which bend and distort the nucleic acid in a profoundly different way to the wild type, it is unlikely that simple alteration of amino acids will lead to any major variations in cleavage specificity.

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BI9824893