The dependence of DNase I activity on the conformation of oligodeoxynucleotides

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We have developed a sensitive continuous assay for nucleases using proton release. The assay has been applied to the determination of the kinetics of DNase I acting on short, defined deoxyoligonucleotides. The dependence of k_{cat}/K_m on sequence and structure of short oligonucleotide substrates has been measured: increasing lengths of A_nT_n sequences decrease the rate of cleavage. G·A mismatches in which the bases pair using imino protons are cleaved quite effectively by DNase I. In contrast, tandem G·A mismatches which use amino pairing and have B_{II} phosphodiester, are refractory to DNase I. Also, the

DNA strands of DNA·RNA hybrid duplexes are not cleaved by DNase I. These results show that the global conformation of a duplex and the details of its minor groove affect the cleavage efficiency by DNase I. The assay has also been used to measure the inhibition constant of the minor-groove-binding ligand propamidine. A value of $3 \mu\text{M}$ has been determined for binding to the sequence d(CGCGAATTCGCG)₂, showing that dissociation constants can be determined even when there are no convenient optical signals for titrations.

INTRODUCTION

Nuclease activity is commonly assayed by counting radioactively labelled products that are separated by gel electrophoresis [1,2] or chromatography [3]. This is an intrinsically discontinuous assay method and can be a lengthy and tedious process. The main advantages are high sensitivity and requirement for small amounts of material, which may be especially important for RNA-processing enzymes. It is also possible to measure non-specific nuclease activity by monitoring degradation of polymeric material at 260 nm, using the hyperchromicity, such as DNase I assays [4]. However, in this case it is usually not possible to determine absolute rates in terms of product concentration. A third technique relies on measuring protons on hydrolysis, which has been done for ATPases using a pH-electrode [5], or with indicators as applied to dehydrogenases [5,6]. Both methods are continuous, and provide a measure of the number of bonds cleaved per unit time. We have used the last of these methods to determine the structural factors that determine the specificity of the nuclease DNase I from bovine pancreas.

DNase I (EC 3.1.21.1) is a non-specific nuclease that acts from the minor groove and cleaves phosphodiester bonds on one strand at a time. It has been suggested that the catalytic efficiency is sensitive to the width of the minor groove [1,7]. The minor-groove width has been shown to be narrower in A-tracts of the kind $dA_n \cdot dT_n$ than in DNA of mixed sequence [8]. In principle, the width of the minor groove can be modulated by changing the DNA sequence, and can therefore be used to probe the characteristics of DNase I in greater detail. Furthermore, the conformation of the phosphodiester backbone is distorted by mismatched base-pairs, especially the tandem G·A base-pair in the context YGAR [9–12] (Figure 1). Such mismatched DNA sequences provided as substrates for DNase I can be therefore be used to probe the dependence of the catalytic efficiency on phosphodiester conformation in greater detail. Although DNase

I is non-specific, it does not cleave RNA. However, a DNA·RNA hybrid in principle might be a substrate for the enzyme, as the conformation of the deoxynucleotides is similar to that in DNA

Figure 1 Base-pairing in G·A mismatches

(A) Imino pairing found in RGAY; (B) amino pairing found in YGAR.

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duplexes [13,14], whereas the overall conformation is closer to the A form of RNA than the B form of DNA. Such duplexes can therefore be used to determine the dependence of DNase I on global conformation.

The preferred site size of DNase I is 4–6 bp [7]. Hence, a short deoxynucleotide (≈ 12 bp) contains only a few potential cleavage sites. Further, the products of the reaction will be too short (≈ 6 bp) to act as good substrates for the enzyme. We have used a spectrophotometric method for measuring the activity of DNase I against defined oligonucleotides to test the structure-sensitivity of the enzyme in detail.

It was also expected that minor-groove-binding ligands, such as the diamidines, which prefer AATT stretches of DNA, would be competitive inhibitors of the enzyme [2]. By measuring the kinetic parameters as a function of ligand concentration, the inhibition constant can be determined, which for many of these ligands is not possible using optical signals [15,16].

EXPERIMENTAL

Materials

DNase I type IV and calf thymus DNA were purchased from Sigma (Poole, Dorset, U.K.) and used without further purification. The following defined HPLC-purified oligonucleotides were purchased from Oswel:

CG6: d(CGCGCGCGCGCG)

AT6: d(ATATATATATATAT)

A2T2: d(CGCGAATTCGCG)

A3T3: d(CGCAAATTTGCG)

A4T4: d(CGAAAATTTTCG)

T2A2: d(CGCGTTAACGCG)

YGAR: d(CGCGCGAGCGCG)

RGAY: d(CGCGGGACCGCG)

GA10: d(GAGTGAACGA)

dRdY: d(GAAGAGAAGC)·d(GCTTCTCTTC)

rR·dY: r(GAAGAGAAGC)·d(GCTTCTCTTC)

dR·rY: d(GAAGAGAAGC)·r(GCUUCUCUUC)

Cresol Red was purchased from Fluka and used without further purification. Propamidine was a gift from Dr. T. C. Jenkins and had been synthesized and purified as described in [17]. All other materials were of analytical grade.

Kinetics were measured in a buffer consisting of 100 mM KCl, 1 mM NaHCO₃, 100 μ M CaCl₂ and 5 mM MgSO₄, pH 8. Cresol Red was also present at 20 μ M.

Methods

Oligonucleotides were dissolved in the assay buffer and annealed by slow cooling from 80 °C. Concentrations were determined from the absorbance at 260 nm and the absorbance coefficients calculated from the nucleotide composition [18] and the absorbance of a solution before and after exhaustive digestion with nuclease P1. In general, calculated duplex absorption coefficients agreed with the measured ones to within better than 5%, and the worst to within 9%. Stock solutions were typically 200–300 μ M duplex. Absorption coefficients for dRdY, rRdY and dRrY were also determined by nuclease P1 digestion [21].

The hydrolysis of the phosphodiester bond is accompanied by the release of a proton:

This reaction is shown leading to a 3'-phosphate monoester, but the same result is obtained if the reaction leads to a 3'-hydroxy plus a 5'-phosphate. The release of the proton is stoichiometric with bond cleavage if the pH of the solution is well above the pK of the terminal phosphate, which is typically 6.6–6.7 [19]. At pH 8, 0.95 mol of H⁺ is released/mol of phosphodiester bonds hydrolysed, and therefore detection of the released proton provides a direct measure of bond cleavage.

We have used a spectrophotometric method with an indicator (Cresol Red) for measuring proton release. The absorbance of the indicator solution is given by the Beer–Lambert law:

$$A = \epsilon_b[B] + \epsilon_a[A] \quad (2)$$

where ϵ_b and ϵ_a are the absorption coefficients of the base (B) and acid (A) forms respectively. For a total concentration of indicator $[B]_t (= [A] + [B])$, eqn.(2) becomes:

$$A = \epsilon_b[B]_t + (\epsilon_a - \epsilon_b)[A] \quad (3)$$

Under conditions where $[B]_t$ is much greater than K , the binding of protons to B will be stoichiometric. The total proton concentration, $[H]_t$, will be the sum of the free protons and those in the acid form of the indicator. Using the conservation equations:

$$[A] = 0.5([H]_t + [B]_t + K - \{([H]_t + [B]_t + K)^2 - 4[H]_t[B]_t\}^{0.5}) \quad (4)$$

A change in $[H]_t$ due to proton release will lead to a change in $[A]$. Because $[B]_t$ and therefore $[H]_t$ are much higher than K , the square-root term in eqn. (4) simplifies to $[B]_t - [H]_t$ and the derivative $d[A]/d[H]_t = 1$. Hence the change in $[A]$ for a change in $[H^+]$ is simply $\Delta[A] = \Delta[H^+]$. The absorbance varies linearly with the number of protons released and is proportional to the difference absorption coefficient. Sensitivity is maximized by choosing an indicator that has a very large difference coefficient and an appropriate pK value. Cresol Red has a difference absorption coefficient of 50 mM⁻¹·cm⁻¹ at 572 nm and a pK value of 8.1. In practice, there will be additional buffering capacity present, such as from dissolved CO₂, which competes for released protons, so that proton binding by the indicator is no longer stoichiometric. The influence of solution buffering capacity was determined by making up solutions of indicator in the assay buffer, and titrating with small aliquots of standard HCl or NaOH. Under these conditions, 1 μ M H⁺ added caused a change in A_{572} of 0.0083, which is equivalent to an effective absorption coefficient of 8300. The change in absorption was linear over a range of more than 20 μ M added acid.

Steady-state kinetics were made on a Hitachi U-3210 spectrophotometer equipped with a circulating water bath. Temperature was maintained at 298 K, and monitored with a thermocouple on the cuvette block. The drift rate was determined as < 0.0001 A/min, and peak-to-peak noise at 572 nm was < 0.001 A. Reliable rates of 0.001 A₅₇₂/min could be determined under these conditions. Kinetics were carried out in 1-cm-pathlength cuvettes over a wide range of substrate concentrations, in triplicate. Reactions were initiated by addition of a small volume (typically 5 μ l) of enzyme solution to the substrate solution, such that the total substrate concentration greatly exceeded the enzyme concentration. Initial rates were determined from the progress curves and converted into concentration units as described above. Kinetic parameters were determined by linear regression to the equation:

$$d[P]/dt|_0 = v_0 = k_{\text{cat}} \cdot [E]_t[S]_t/K_m \quad (5)$$

where $[E]_i$ and $[S]_i$ are the initial enzyme and substrate concentrations respectively.

Inhibition by propamidine was determined by measuring the rate of the reaction as a function of increasing concentration of inhibitor. The data were analysed according to the uncompetitive model:

Scheme 1

where D, E, L and P represent DNA, enzyme, propamidine and product respectively, and K_i and K_i' are dissociation constants for the inhibitor complexes.

The steady-state initial velocity for this model is:

$$v_0 = k_{cat} / (1 + [L]/K_i') [E]_i \cdot [D] / \{ [D] + K_m / (1 + [L]/K_i') \} \quad (6)$$

where $[D]$ and $[L]$ are the free concentrations of DNA and ligand respectively. The concentration of free DNA decreases as the concentration of the inhibitor is increased. As will be shown below, the K_m for DNase I is very high, and the observed rate is strictly proportional to the concentration of DNA, i.e. K_m is much greater than $[D]$. In this case, eqn. (6) becomes:

$$v_0 = k_{cat} [E]_i \cdot [D] / K_m \quad (7)$$

Under the conditions where $[L]_i$ and $[D]_i$ are greatly in excess of the enzyme concentration, such that $[D]_i \approx [D] + [DL]$ and $[L]_i \approx [L] + [DL]$, then $[D]$ can be calculated according to:

$$[D] = -([L]_i - [D]_i + K_i) + \{ ([L]_i - [D]_i + K_i)^2 + 4K_i[D]_i \}^{0.5} \quad (8)$$

k_{cat}/K_m was measured as a function of propamidine concentration, and the parameters k_{cat}/K_m and K_i were determined by non-linear regression to eqns. (7) and (8). To show that propamidine does not inhibit the enzyme directly, the substrate CG6 was used for a control, as it does not significantly bind the ligands under the conditions of the experiments (and see below). For the substrate AT6, there was residual cleavage at saturating concentrations of propamidine, attributable to the presence of multiple overlapping sites (see the Results section). We have therefore also used a modification of eqn. (7) as follows:

$$v_0 = k_{cat} [E]_i \cdot [D] / K_m + v' \quad (9)$$

where v' represents the residual rate at saturation with ligand.

RESULTS

Action of DNase I on polymeric calf thymus DNA

To verify that DNase I activity can be adequately monitored by proton release, we first used calf-thymus DNA as the substrate, as this is similar to natural substrates and can be prepared in high concentration. When the hydrolysis reaction was monitored at 260 nm in the absence of Cresol Red (using the hyperchromicity), there was an initial fast increase in absorbance, followed by a slower phase (not shown). Similar traces were observed in the presence of Cresol Red at 572 nm (by proton release), but of opposite sign and lower amplitude. The initial fast phase corresponds to rapid cleavage of the large fragments, and is followed by slower turnover and probable substrate inhibition by smaller fragments and decreasing pH. The dependence of the

Figure 2 Dependence of rate of cleavage of calf thymus DNA on DNase I

Unfractionated calf thymus DNA was prepared as described under 'Methods', and cleavage by DNase I was monitored at 260 nm (—Cresol Red) or at 572 nm (+Cresol Red). $\square$, A_{260} ; $\blacksquare$, A_{572} . (A) Dependence of the initial rate on the concentration of DNase I; (B) dependence of the initial rate on concentration of base-pairs at $0.5 \mu\text{M}$ DNase I.

initial slope of the reaction on enzyme concentration is shown in Figure 2(A) and on the concentration of DNA in Figure 2(B). The reaction rate is directly proportional to the enzyme concentration, measured either by hyperchromicity or by proton release. Further, the rate increases linearly with increasing DNA concentration up to at least $60 \mu\text{M}$ base-pairs, indicating a high K_m for this substrate. The rate when expressed as absorbance units/time is larger for hyperchromicity than proton release. However, it is not possible to convert changes in A_{260} into concentration of product (or mol of bonds cleaved), whereas the absorbance at 572 nm can be expressed as mol of bond cleaved per unit time (see under 'Methods').

Difference spectra of enzyme $\pm$ Cresol Red and DNA $\pm$ Cresol Red were flat, consistent with insignificant binding of the indicator to either enzyme or substrate. Further, the rate of the reaction was independent of the concentration of Cresol Red, confirming that the indicator is not an inhibitor of the enzyme over the concentration range under investigation. Because calf thymus DNA is polymeric, the observed rate is clearly an average of all possible sequences. These results nevertheless establish that the proton-release assay faithfully measures the cleavage of the DNA substrate and, moreover, provides quantitative information about the number of bonds cleaved.

Action of DNase I on defined oligonucleotides

Undefined polymeric DNA, such as calf thymus DNA, can provide only averaged kinetic parameters. We have used defined

Figure 3 Dependence of cleavage rates by DNase I on concentration of different substrates

Initial rates were measured using proton release, and the rate was converted into mol of bonds cleaved as described under 'Methods'. ●, T2A2; ■, A2T2; ▲, A3T3; □, RGAY; ○, YGAR.

Table 1 Steady-state kinetic parameters for DNase I at 298 K and at pH 8

k_{cat}/K_m was calculated on the basis of the duplex concentration. The calculated ϵ value is the absorption coefficient calculated from mono- and di-nucleotides, and the observed ϵ value is the absorption coefficient determined from digestion with nuclease P1 as described in the Experimental section. Abbreviation: nd, not determined.

Substrate	ϵ (mM ⁻¹ ·cm ⁻¹)		No. of bp	$10^{-3}k_{cat}/K_m$ (M ⁻¹ ·s ⁻¹)
	Calculated	Observed		
CG6	165.2	160.8	12	4.8
AT6	217.8	202.8	12	6.0
T2A2	184.8	181.5	12	6.66
A2T2	184.0	182.6	12	5.16
A3T3	187.2	176.7	12	2.82
A4T4	197.2	nd	12	1.98
RGAY	180.0	187.2	12	5.0
YGAR	178.4	182.8	12	1.9
GA10	165.0	150.9	10	< 0.01
CT*	—	—	—	2.1†
dR.dY	153.0	146	10	0.94
dR.rY	157.4	149	10	0.01
rR.dY	153.0	154	10	< 0.01

* CT calf thymus DNA.

† For base-pair concentration.

substrates to determine the kinetic parameters as a function of sequence and structure. Figure 3 shows the dependence of the initial velocity on the concentrations of several substrates. No deviations from linearity were observed up to 14 μ M total oligonucleotide concentration, which indicates K_m values greatly in excess of 14 μ M. In fact, the concentration of sites is at least twice the oligonucleotide concentration as they are palindromes, and also presumably contain several potential sites if the minimum target size for DNase I is of the order of 6 bp [7]. Hence only the parameter k_{cat}/K_m can be reliably determined in this system. The values are given in Table 1, which shows that k_{cat}/K_m is rather low, consistent with an enzyme of low specificity and low overall activity. For comparison, k_{cat}/K_m for the *Eco*RI restriction endonuclease is $\approx 10^6$ M⁻¹·s⁻¹ at 20 °C [3].

There are significant differences in the specificity parameter for

the various substrates, which can be ranked in the following order: T2A2 > AT6 > A2T2 > RGAY > CG6 > A3T3 > A4T4 $\approx$ YGAR $\approx$ GA10 (Table 1). Comparing T2A2 with A2T2, A3T3 and A4T4, it is apparent that the activity decreases as the length of the A_nT_n tract increases, with a variation of about fourfold between T2A2 and A4T4. This is probably related to the different structural characteristics of the A_nT_n sequences compared with T2A2, which does not have a narrowed minor groove [1,8].

The RGAY mismatch, where the G and A residues pair using the imino protons, is a much better substrate than the YGAR and GA10 oligonucleotides where the bases pair using the amino protons (Figure 1) [9–12], and the backbone position and the nature of the minor groove are very different from that in standard B-DNA. This indicates that the perturbation of the backbone in the imino-paired tandem GA mismatch has only marginal influence on the rate of the DNase I, whereas the amino-paired tandem GA mismatch causes a greater distortion, particularly in the minor groove, and reduces the rate substantially. This is further demonstrated by the GA10 sequence which consists almost entirely of blocks of nucleotides having a non-standard B conformation [11]. Activity was essentially undetectable with this sequence. The sizes of the products were estimated from analysis by electrophoresis. The cleaved products consist of a group of poorly overlapped bands on gels indicating sizes between 5 and 9 bp. This is consistent with a preferred DNase I site of around 6 bp, so that the dodecamers actually contain about six potential cleavage sites. Hence, the observed rates of cleavage reflect an average over the microsequences in the centre of the molecule. For this reason, the differences in the observed rates probably underestimate the intrinsic cleavage rates for individual dinucleotide steps.

In addition, the total absorbance change at $t = \infty$ was measured and converted into mol of cleaved bonds. On allowing the reaction to go to completion, approximately three bonds per duplex were cleaved for T2A2, two for A2T2 and CG6 and one for A3T3 and RGAY. This indicates that, for the first three duplexes, the initial nicked products of the reaction are still substrates, whereas for the last two, the nicked products do not act as substrates.

Action of DNase I on DNA·RNA hybrids

The results presented above on the action of DNase I on different sequences, and the effects of certain mismatches, show that the enzyme has some structural requirements: it will cleave only double-stranded DNA, and defined distortions in the minor groove significantly affect its ability to cleave (Table 1). The enzyme is inactive against RNA, which is in the A-form, and has a very wide, shallow minor groove. We wondered whether a DNA·RNA hybrid would be a substrate for the enzyme, because, whereas RNA·DNA hybrids are globally in the A conformation, the deoxynucleotides are in a B-like conformation [13,14,20,21]. We have therefore compared the activity of DNase I on the DNA duplex d(GAAGAGAAGC)·d(GCTTCTCTTC) with its activity on two DNA·RNA hybrids of similar base sequence (cf. the 'Methods' subsection). The results are summarized in Table 1. The cleavage rates against the two hybrids were much lower than the parent DNA duplex, and the residual activity was < 1–2%. This indicates that DNase I shows a preference for the global B-conformation. Also, the DNA nucleotides in DNA·RNA hybrids are more flexible than in DNA·DNA duplexes [14]. This feature is particularly marked in the hybrid duplexes used here, which also showed global conformations different from those of the DNA duplex [21]. The additional flexibility may also play a role

Figure 4 Inhibition of DNase I cleavage by propamidine at 298 K and pH 8

The initial rate of the DNase I reaction was measured by proton release at 2–3 μM DNA substrate as a function of the concentration of propamidine. Lines are best-fit curves to eqns. (7) and (8) as described in the text. $\square$, CG6; $\blacksquare$, A2T2

Table 2 Inhibition constants for propamidine

Inhibition constants (K_i) were determined at 298 K and at pH 8, as described in the text.

Sequence	K_i (μM)
CG6	> 30
A2T2	3 ± 0.5
A3T3	≈ 1
AT6	$10 \pm 3^*$

* Effective K_i (see the text).

in determining the cleavage rate (see below). The DNA duplex was cleaved at a low rate compared with the other DNA duplexes (Table 1), though this may be attributable to the unusual base composition of the two strands. Furthermore, as this duplex (10 bp) is shorter than most of those in Table 1 (12 bp), it has fewer potential cleavage sites.

Inhibition of DNase I by propamidine

Propamidine is an aromatic bisamidinium compound that selectively binds to the minor groove of DNA at AT stretches. The crystal structures of propamidine complexed with A2T2 and A3T3 have been determined [22,23], and the solution structure has been determined with an asymmetric decamer containing the subsequence AAT [24]. The ligand occupies 4–5 bp in the AATT regions of the molecule. Footprinting experiments have shown that propamidine and the related ligand berenil decrease the cleavage probability of DNase I [2]. Therefore, we have used the present assay to characterize this interaction in greater detail.

Figure 4 shows the dependence of the initial rate of cleavage of A2T2 and CG6 on the concentration of propamidine. For both A2T2 and A3T3 (results not shown), k_{cat}/K_m decreased nearly 10-fold over the range of ligand concentrations from 0 to 30 μM , whereas for CG6, the rate decreased by $< 20\%$. This shows that the inhibition arises from competition with the DNA, rather than from interaction with the enzyme. K_i values were determined for each substrate as described in the Experimental section and are given in Table 2. The inhibition constant for A2T2 is 3 μM , whereas that for A3T3 is about 1–2 μM . These values are comparable with dissociation constants measured optically for

berenil binding to various DNA species [15,16,24]. The lack of inhibition of the reaction with CG6 ($K_i > 30 \mu\text{M}$) indicates an AT preference of at least 10-fold for this ligand, in agreement with other studies [25].

In AT6, there are in principle several overlapping binding sites for propamidine, and occupation of only one site may leave DNase I cleavage sites available. Hence the apparent inhibition constant measured by this method is an average and, as such, gives a lower limit to individual site constants. Furthermore, even at saturation, residual cleavage may occur. We have therefore modified the fitting equation to include a constant term (v') for residual cleavage (eqn. 9). Nevertheless, the apparent K_i was substantially larger than observed for A2T2 or A3T3, showing that propamidine binds relatively weakly to sequences of the kind ATAT or TATA.

Including the residual term for A2T2 had no influence on the fit, and the fitted value of v' was not statistically different from zero. This indicates that occupation of the AATT site leaves no DNase I cleavage sites vacant. This is consistent with a minimum binding site for DNase I of at least 4 bp, i.e. the terminal 4 bp do not act as good substrates for the enzyme.

The very low rate of cleavage of the DNA·ligand complex (Figure 4) could arise either because the enzyme has insignificant affinity for the complex (i.e. K_i' is very high), or simply because the ligand prevents access to the cleavage site of the DNA. The same can be argued for GA10, where the minor groove is filled with the edges if the mismatched bases. If the latter is true, then GA10 should act as a simple competitive inhibitor, and therefore decrease k_{cat}/K_m for a 'good' substrate. We have measured the rate of cleavage of A2T2 at a fixed concentration in the absence and presence of different concentrations of GA10. No inhibition was observed up to 25 μM , indicating an inhibition constant (K_i) much greater than 25 μM .

DISCUSSION

There are three main ways of measuring proton release, all requiring the use of unbuffered solutions. First, a sensitive pH-electrode could be used to measure the change in pH [5]. Secondly, a pH-stat apparatus could be used to maintain the pH at a constant value, which provides an automatic read-out of the concentration of hydroxide added per unit time [4]. The third method is spectrophotometric, whereby the released proton binds to an indicator having the appropriate p*K*, and which has a very large difference absorption coefficient between the base and acid forms. We have used the third of these methods and have applied it to the kinetics of DNase I acting on short, defined sequences of synthetic DNA.

The major advantage of the proton release assay is that it is continuous and therefore requires no additional work-up. It is also rapid and, provided that a stable spectrophotometer is available, sensitive. Because the proton release can be quantified, the number of bonds cleaved can be calculated, which is an advantage compared with using hyperchromicity. Proton release is of course only effective for pH values above the p*K* value of the product phosphate monoester (≈ 6.7), and is therefore not usable for quantitative assays of nucleases having low pH optima (e.g. nuclease P1). The resolution for non-specific nucleases is not as high as with the discontinuous assay employing gel electrophoresis and footprinting conditions [1,2], where single-base resolution is attained. However, the quantification by that process is more difficult, and the method is not particularly suited to determining dissociation constants for small ligands.

We have confirmed that DNase I is sensitive to structure in the minor groove. Thus increasing lengths of A_nT_n tracts decrease

the cleavage efficiency. Drew and Travers reported that A_nT_n tracts were especially poorly cleaved by DNase I [1], and our results are in accord with their findings. They noted that such sequences are associated with a decrease in the width of the minor groove, which has been subsequently confirmed by further structural work [8,22,23]. However, A_nT_n tracts are also more rigid than mixed-sequence DNA [8,19]. This is consistent with the free-energy change for melting of such sequences, which increases in the order $d(CGCT3A3GCG)_2$ [255.2 kJ (61.0 kcal)·mol⁻¹] < $d(CGCATATATGCG)_2$ [320.1 kJ (76.5 kcal)·mol⁻¹] < $d(CGCA3T3 GCG)_2$ [341.0 kJ (81.5 kcal)·mol⁻¹] (G. L. Conn and T. Brown, unpublished work). Furthermore, the TpA step in T2A2 sequences is especially flexible [26,27]. Thus the efficiency for DNase I is negatively correlated both with minor-groove width and structural rigidity. The importance of the minor groove is also shown by the results obtained with the tandem G·A mismatches. In the YGAR motif, in which the mismatched pair is in the amino form (Figure 1), the bases fill the minor groove and also have rather different phosphodiester conformations compared with B-DNA [9–12]. These sequences are not substrates for DNase I. The filling of the minor groove may account for the inhibition by propamidine, which slots into the minor groove and would prevent access to groups within the groove [15,16,22–24]. Furthermore, DNase I was inactive against the two DNA·RNA hybrids, which are globally closer to the A-form than to the B-form, even though the nucleotides in the DNA strand are in a conformation similar to that found in DNA duplexes [13,14,20,21]. In these duplexes, the minor groove is wide and shallow. This indicates that DNase I has significant conformational specificity in that it prefers double-stranded DNA duplexes in which the minor groove is unobstructed, neither too narrow nor too wide, and where the duplex retains significant flexibility.

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