Protection of (dA·dT) cluster regions in the DNAase I cleavage of DNA by specific interaction with netropsin

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Received 27 May 1980

ABSTRACT

The specific DNA binding ligand netropsin selectively blocks dA·dT base pairs in clusters containing two or more consecutive thymine residues at the DNAase I cleavage sites of DNA. Using CD and UV absorption measurements it is shown, that at various ratios of netropsin to nucleotide concentrations and even at saturation of ligand interaction the enzyme cuts along regions containing dG·dC pairs sandwiched between dA·dT pairs. This follows a slow kinetics and is associated with a release of netropsin from those segments. These facts suggests the usefulness of the partial protection of certain DNA sequences in DNAase I cleavage sites in producing DNA fragments in structural studies of the genome. A possible interpretation of the effect of netropsin binding on the enzymatic hydrolysis of phosphodiester bonds of the helix is discussed.

INTRODUCTION

The oligopeptide antibiotic netropsin (Nt) specifically binds to dA·dT base pairs in the minor groove of duplex DNA¹⁻⁵ with high preference to nucleotide clusters⁶ containing two or more consecutive thymine residues⁷. It has been widely demonstrated that this specific ligand interaction may be used to selectively block the action of DNA enzymes at discrete regions of the template (reviewed in ref. 1) such as DNA polymerases⁸⁻¹¹, RNA polymerases⁹⁻¹³ or restriction endonucleases^{14,15}. Recent findings have shown that Nt¹⁴⁻¹⁶ or distamycin (Dst-A)¹⁷⁻¹⁹ together with actinomycin D^{15,16,18,19} are most effective inhibitors of restriction endonucleases, which may be employed for a partial protection of their specific cleavage sites due to the base specificity of the DNA binding ligands. The partial protection of endonucleases cleavage regions of the DNA is of general importance with respect to

an increase of their selectivity in producing DNA fragments and for the characterization of the nucleotide environment of the cleavage pattern. These aspects are of interest in the investigation of the genome structure and functions.

In the present study we describe first results of a selective blocking of (dA·dT) rich nucleotide regions in the DNAase I cleavage sites of DNA by Nt.

MATERIAL AND METHODS

Nt hydrochloride used in these experiments was isolated from Streptomyces netropsis by H. Thrum². Extinction coefficients are $\epsilon_{238} = 19800$ and $\epsilon_{296} = 21500$ ¹. The following extinction coefficients were used for DNA's: Calf thymus DNA (CT), $\epsilon_{260} = 6500 \text{ M}^{-1}\text{cm}^{-1}$; Sarcina maxima DNA (SM), $\epsilon_{260} = 6500 \text{ M}^{-1}\text{cm}^{-1}$; Streptomyces chrysomallus DNA (SC), $\epsilon_{260} = 6200 \text{ M}^{-1}\text{cm}^{-1}$; values of synthetic duplex polymers (from Boehringer, Mannheim, G.F.R.) are those previously used⁷.

DNAase I from Novo (Copenhagen) or Ferrak (Berlin) was employed in these studies. Enzymes of different origin (Boehringer, Mannheim) showed the same effects. Incubation mixtures contained 0.02 M Na acetate, 5 mM MgCl_2 (pH 5.5) at a DNAase I concentration of approx. 5 $\mu\text{g/ml}$ if not otherwise mentioned. Freshly prepared enzyme solutions (3 to 4 mg enzyme/ml) were used for rapid mixing procedure. Standard reactions are carried out at DNA-P concentrations $1 \times 10^{-4} \text{ M}$ - $2 \times 10^{-4} \text{ M}$.

CD spectra were recorded in a Cary 60 spectropolarimeter with CD attachment using 1 cm cells and a special device allowing rapid mixing for measurements of slow kinetic experiments. CD data are given as $\Delta\epsilon = \epsilon_L - \epsilon_R$.

UV absorption measurements were carried out in a Uvispek spektrophotometer. Mixing of the enzyme added in CD and UV absorption measurements was performed within one to five seconds in a special procedure using 1 cm cells so that the initial reaction monitored by the extinction was close to the extinction at zero time without enzyme.

RESULTS

CD measurements: CD spectral changes directly reflects specifically bound Nt to duplex DNA by the extrinsic Cotton effect outside the DNA spectral region around 315 nm (zero-time spectra Fig. 1). Since Nt probes the DNA duplex structure 1-4 DNAase I cleavage followed by opening of double-stranded regions and formation of single-stranded segments should result in the re-

Figure 1: Effect of DNAase I on the spectra of netropsin complexes with different DNA's at a ratio of Nt/DNA-P = 0.1 and 0.15. A, poly(dA)·poly(dT); B, CT DNA (58 mole% A + T); C, SCDNA (28 mole% A + T); attached numbers indicate time in minutes.

lease of Nt and a lowering of the characteristic CD band around 315 nm. In fact we found that DNAase I causes a decrease of the characteristic CD band due to cleavage of the DNA as demonstrated in Fig. 1 for DNA from calf thymus and from Streptomyces chrysomallus. The dissociation of Nt from DNA occurs with slow kinetics approaching more or less a constant low value of the ellipticity after 40 to 50 minutes (Fig. 2). Fig. 1 shows a progressive alteration of the CD spectrum of the Nt-DNA complex into a spectrum indicating strongly reduced Nt binding after 40 minutes in the case of DNAase I action on calf thymus DNA. This demonstrates a partial protection of DNA segments by Nt binding. The release of Nt from the (dG·dC) rich DNA complex is almost completed after 40 minutes (Figs. 1 and 2) and hence this DNA is almost not protected by Nt interaction. On the contrary at comparable ratio of moles Nt/nucleotide, $r' = 0.1$, the Nt poly(dA)·poly(dT) complex shows practically no change in the CD spectrum reflecting complete protection of this duplex polymer by bound Nt. These results indicate an increase in the stability

Figure 2: Kinetics of DNAase I cleavage of the Nt-DNA complexes by monitoring the release of Nt reflected at the CD maximum at 315 nm. Attached numbers indicate ratio Nt/DNA-P:

of the Nt-DNA complex against the attack of the enzyme with increasing dA:dT content.

UV absorption measurements: DNAase I cleavage of DNA is generally monitored by UV absorption measurements. To investigate directly phosphodiester bond hydrolysis of the DNA in the Nt complex on the basis of UV absorbance changes at 260 nm, measurements have to be corrected owing to an absorbance contribution of released Nt in this spectral range. DNA complexed with Nt shows a hypochromic effect relative to DNA (1,2). As an example Fig. 3 indicates a progressive increase in the extinction at 300 nm of the DNA-Nt complex on DNAase I cleavage at different relative concentrations of Nt. Since at this wavelength DNA almost does not contribute to

Figure 3: Kinetics of the Nt release from the DNA complex in the course of DNAase I hydrolysis of DNA measured by variation of the absorbance at 300 nm; numbers designate ratio Nt/DNA-P.

the absorption of the complex the observed ΔE values directly reflect released Nt nearly without structure effects of the DNA and hence may be used for corrections at 260 nm. Using repeatedly estimated absorbance ratios of $R = E_{300}/E_{260}$, the apparent enzyme cleavage monitored at the absorption maxima of various DNA's, was corrected on the basis of simultaneous measurements at 300 nm: $E_{\text{corr.}(260)} = E_{\text{app.}} - \Delta E(300) \cdot R$.

The extent of DNA cleavage depends on DNA concentration of free DNA and in the complex at 2 minutes but becomes independent at higher cleavage time (results not shown). The results for the kinetics of the DNAase I cleavage of DNA in the presence of increasing Nt to DNA phosphate ratio are shown in Fig. 4. It may be seen, that increasing Nt concentrations

Figure 4: Kinetic of the DNAase I cleavage of the DNA-Nt complex monitored by UV absorbance changes at 260 nm

partially protect DNA regions to higher extent in the case of the (dA·dT) rich CT DNA whereas DNA of high dG·dC content is almost degraded to similar extent in the complex as in free DNA. The small inconsistencies of short cleavage steps observed in the kinetic curves of natural DNA's (Fig. 4) are not clear as yet, but the general course of the curves are, however, not affected. These data again demonstrate the dependence of the protective action for the DNAase I cleavage sites by Nt on base composition indicating a selectivity for enzyme blocking in (dA·dT)rich regions.

The highly selective protection of certain DNA sequences is more clearly evidenced by the kinetic data in Fig. 5. There is a different dependence of the inhibition effect on the Nt concentration. Poly(dA)·poly(dT) is completely protected at a ratio of Nt $r' = 0.1$ whereas a higher concentration, $r' = 0.2$ is required for a total inhibition of the DNAase I

Figure 5: Kinetics of the DNAase I cleavage of Nt complexes with synthetic DNA duplex polymers monitored by UV absorbance changes at the maxima of polymers; numbers indicate ratio Nt/nucleotide

activity in the case of poly(dA-dT)·poly(dT-dA). It is very striking that poly(dA-dC)·poly(dT-dG) is almost not protected at comparable ratio of Nt to nucleotide and even not at $r'=0.4$. This means insertion of one dG·dC pair between two dA·dT pairs forms Nt binding regions which are sensitive for the action of the enzyme as in non-complexed DNA. This may be explained by a lower binding constant as considered below. Similar behaviour occurs in the cleavage kinetics of poly(dG-dC)·poly(dC-dG) (Fig. 5) which does not specifically bind Nt^{1,3-5}. A plot of the percentage of the Nt protected fraction of the DNA (Fig. 6) versus ligand concentration demonstrates that under the conditions used a high specific effect exists of Nt in protecting a DNA sequence containing dA·dT base pair clusters evidenced by the variation of the effective blocking concentration range.

DISCUSSION

The CD spectral changes and kinetics shown in Figs. 1 and 2 reflect removal of Nt from the DNA complex as a result

Figure 6: Dependence of the netropsin induced inhibition of DNAase I hydrolysis of DNA's on the ligand concentration. Fraction of DNA blocked refers to Fig. 5

of DNAase I cleavage of the phosphodiester bond while the effect of Nt binding on the DNAase I digestion of DNA is directly indicated in the kinetic studies in UV absorption measurements as displayed in Figs. 3 to 5. Two main effects are visualized: protection of (dA·dT) rich regions and occurrence of DNA cleavage within regions containing dG·dC pairs interspersed between dA·dT pairs.

In particular digestion experiments with sequential DNA duplex polymers (Figs. 5 and 6) demonstrate that Nt binding specifically protects dA·dT pairs in clusters. On the basis of these kinetic results we may suggest a decreasing order for the selectivity of protected nucleotide sequences in the DNAase I cleavage of DNA as presented in Fig. 7. The ability of Nt in blocking sequences containing dA·dT pairs reflect the strong type of Nt binding sites whereas sequences with dG·dC sandwiched between dA·dT pairs represent weak Nt binding sites. These effects are easily explained by the specific interaction of Nt with dA·dT pairs found for different DNA's and synthetic duplex polymers¹⁻⁷. Strong DNA binding regions related to dA·dT clusters are represented by a Nt binding constant K of the order of $\sim 10^8 \text{ M}^{-1}$ according to that reported for poly(dA-dT)·poly(dT-dA)²⁰. The overall binding constant estimated for several DNA's are of the order of $K_a \sim 10^5 \text{ M}^{-1}$ to

Figure 7: Scheme of the order of selection of protected nucleotide sequences in DNA by netropsin interaction

1) Suggested not measured

2) Measured at enzyme concentration of $10 \mu\text{g/ml}$.

10^6M^{-1} ^{3,4}. Therefore these differences in the binding constant amounting to two or three orders of magnitude may be responsible for the deviation in the enzymatic cleavage activity between strong and weak Nt binding regions in DNA. In the case of poly(dG-dC)·poly(dC-dG) and of dG-dC clusters in DNA Nt does not show any specific binding effect¹⁻⁵ and hence DNAase I cleavage is not greatly affected. The observed sequence dependent protection effect (Fig. 7) closely correlates to the decreasing order of the binding effect observed in CD studies⁷.

The present findings are important with respect to several aspects in the endonuclease digestion of DNA. In both types of binding regions, in dA·dT clusters as well as in domains containing dG·dC pairs interspersed between dA·dT pairs Nt fills the minor groove and hence this will prevent access and recognition of the duplex DNA by the enzyme from this groove. Since DNAase I cuts within the latter sequence complexed with Nt but is inhibited along dA·dT cluster regions one may suggest cleavage of DNA from the major groove.

The high stability of protected dA·dT clusters may be explained by several factors. Interaction of Nt in these regions cause a stiffening^{2,21,22} and possibly a weak conformational change²²⁻²⁵ associated with a maximal lowering in the motional freedom of the isohelically fixed oligopeptide molecule²⁵. This is achieved by formation of three or four hydrogen bonds together with electrostatic interactions so that the covered segment is highly stabilized and modified preventing access of the enzyme. This possible effect is slightly diminished for the alternating dA·dT sequence but is strikingly changed when one dG·dC pair is sandwiched between two dA·dT pairs. The differences in the binding constants discussed above may account for this strong alteration. In particular Nt binding to this sequence is less specific owing to a possible sterical hindrance by the amino group at C(2) of guanosine in the minor groove³ and the associated restricted formation of two possible hydrogen bonds on adjacent dA·dT pairs. This may cause a higher motional freedom of the bound

Nt molecule. Thus unspecific interaction and access of the enzyme with subsequent phosphodiester bond hydrolysis is promoted on a G or C site from the major groove followed by a weakening and removal of the Nt binding site on an adjacent dA·dT pair.

The sequence selective protection of the DNAase I degradation of DNA corresponds to the partial blocking of certain restriction endonuclease sites by netropsin or distamycin¹⁵⁻¹⁸. Our present findings of a selective protection of DNAase I cleavage and the fact that enzymatic hydrolysis proceeds in weak Nt binding regions may therefore imply that those endonucleases could be inhibited by a similar mechanism. The enzymes probably recognize their cleavage site rather from major groove than from the minor groove. This agrees with recent findings on the interaction of the restriction endonuclease HhaI¹⁶ which demonstrated recognition of its cleavage site from the major groove.

Our data may also offer a way for further improvements in enzymatic digestion studies of chromosomes. It will be of interest for possible modifications²⁶ of the banding pattern in combination with DNAase I digestion in the staining procedures of chromosomes.

Acknowledgements: We thank Mrs. Ch. Radtke for excellent technical assistance. The critical discussion and suggestions of Dr. K.E. Reinert is gratefully acknowledged.

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