Oligoamine–acridine conjugates for promotion of gap-selective DNA hydrolysis by Ce(IV)/EDTA complex

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ABSTRACT

Oligoamines (spermidine, dipropylenetriamine and propylenediamine) were covalently attached to acridine via a hexamethylene linker. These oligoamine–acridine conjugates were efficiently bound to gap sites in substrate DNA, and promoted the DNA hydrolysis by a homogeneous Ce(IV)/ethylene-diamine-N,N,N',N'-tetraacetate (EDTA) complex at these sites. In contrast, the hydrolysis of the double-stranded portion in the DNA was little affected by these conjugates, although they were strongly bound thereto by the intercalation of their acridine moieties. As a result, the gap site was selectively and efficiently hydrolyzed by combining the Ce(IV)/EDTA complex with the oligoamine–acridine conjugate. Either the oligoamine or the acridine was only poorly active for the purpose, substantiating the essential role of cooperation between them. The promotion of gap-selective DNA hydrolysis by the conjugates has been ascribed to electrostatic stabilization of a negatively charged transition state by their positive charges.

INTRODUCTION

New tools for site-selective DNA scission are crucially important for further development of molecular biology and biotechnology (1–5). One of their promising applications is the manipulation of DNAs of higher animals and plants, which are too large to be selectively cut by naturally occurring restriction enzymes. Previously (6,7), artificial restriction enzymes were prepared by tethering metal complexes for DNA hydrolysis to the oligonucleotides which are complementary with substrate DNA near the target site. A similar strategy has been employed for the preparation of many ribozyme mimics for RNA hydrolysis (8–16). Recently, a new strategy involving non-covalent fixation of molecular scissors has been proposed (17–19). The target phosphodiester linkage in RNA is differentiated from the other linkages in terms of intrinsic reactivity and preferentially hydrolyzed, although the molecular scissors are freely moving around in the reaction mixtures. For example, the target linkage was activated by non-covalent interactions with acridine-bearing DNA, and was selectively hydrolyzed by various metal ions (19). However, non-covalent approaches to site-selective hydrolysis of DNA have been scarce.

A few years ago, we found that a homogeneous Ce(IV)/EDTA complex efficiently hydrolyzes DNA (20). This scission was further accelerated by spermine and other oligoamines (21). Interestingly, single-stranded DNA was hydrolyzed far more promptly than double-stranded DNA. By using this difference in the reactivity, gap sites in substrate DNA were selectively hydrolyzed by the Ce(IV)/EDTA complex (22). However, this gap-selective DNA hydrolysis is not sufficiently fast and some cocatalysts for its acceleration are desirable.

In order to promote the selective hydrolysis at the gap site, the cocatalysts must fulfill the following requirements: (i) they must be strongly bound to the gap site and (ii) the hydrolysis at the gap site must be accelerated more efficiently than the hydrolysis at the other portions. However, it is not easy to design these cocatalysts, since most of the DNA binders previously reported prefer double-stranded DNA to single-stranded DNA. Although oligoamines are bound to single-stranded DNA, they are also notably bound to double-stranded DNA since the binding is based on simple electrostatic interactions. In this paper, we show that covalent conjugates of oligoamine and acridine are strongly bound to the gap site in DNA substrates and selectively promote the hydrolysis of these sites by a Ce(IV)/EDTA complex. The hydrolysis at double-stranded portions is little affected. Either oligoamine or acridine is inefficient. The interactions of the conjugates with the gap site in DNA are quantitatively analyzed, and the mechanism for the acceleration of gap-selective DNA hydrolysis by the conjugates is proposed in terms of these results.

MATERIALS AND METHODS

Materials

The conjugates 1–3 were synthesized according to the Supplementary Material (Scheme S1), and purified by recrystallization from ethanol/diethyl ether solution. Their structures and purities were confirmed by 1H-NMR. All the
oligonucleotides were prepared on an automated synthesizer and purified by reversed-phase HPLC. Water was deionized by a Millipore Water Purification System and sterilized by an autoclave immediately before use. Commercially obtainable Ce(NH₄)₂(NO₃)₆ (Nacalai Tesque, Inc.) and EDTA·4Na (Tokyo Kasei Kogyo Co., Ltd) were used without further purification.

**DNA cleavage experiments**

The Ce(IV)/EDTA complex was prepared by mixing equal amounts of a 20 mM solution of Ce(NH₄)₂(NO₃)₆ in water and 20 mM EDTA·4Na in HEPES buffer. Then, the pH was adjusted to 7.0 with a small amount of NaOH. The substrate DNA (32P-labeled at the 5′ end) and the DNA additives were incubated in NaCl solution at 95°C for 2 min and slowly (for 15 min) cooled to room temperature. Then, aqueous solutions of 1–3 were added. The cleavage reaction was started by adding an aqueous solution of the Ce(IV)/EDTA complex to the mixture. Typical reaction conditions were as follows: [substrate DNA]₀ = 1 μM, [DNA additive] = 1.1 μM, [Ce(IV)/EDTA] = 500 μM, [oligoamine–acridine conjugate] = 30 μM, [NaCl] = 100 mM, [HEPES] = 5 mM, pH 7.0 and 50°C. After a predetermined time, the reaction was stopped by adding an excess amount of EDTA and inorganic phosphate, and the mixture was subjected to 20% polyacrylamide gel electrophoresis. The quantification of gel patterns was made using a Fuji Film FLA-3000G imaging analyzer.

**Spectroscopy**

Fluorescence, CD and UV/Vis absorption spectra were measured on a FP-750 spectrofluorometer, a J-725 spectropolarimeter and a V-530 UV/VIS spectrophotometer (from JASCO), respectively. Measurement conditions: [NaCl] = 100 mM, [HEPES] = 5 mM, pH 7.0 and 20°C. Scatchard analysis of the binding of 1 to DNAs, based on the neighbor exclusion model of McGhee and von Hippel, was achieved as described in the literature (23).

**RESULTS AND DISCUSSION**

**Gap-selective DNA hydrolysis by the combination of a Ce(IV)/EDTA complex and an oligoamine–acridine conjugate**

In the oligoamine–acridine conjugates 1–3, spermidine, dipropylenetriamine and propylenediamine are bound to
acridine via a hexamethylene linker (Fig. 1a). At pH 7.0, these conjugates have four, four and three positive charges, respectively. The ring-nitrogen atoms of the acridine are mostly protonated [the pKa of the ring nitrogen of 9-amino-6-chloro-2-methoxyacridine is 8.5 (24)] and all the aliphatic amino residues are also protonated. By using appropriate oligonucleotides (Fig. 1b), gap structures of the desired length were formed in the substrate 99mer DNA (32P-labeled at the 5′-end). Figure 2a shows the typical gel electrophoresis patterns for the DNA hydrolysis at 50°C by the combination of the Ce(IV)/EDTA complex and the spermidine–acridine conjugate 1. The results of quantitative analysis of these lanes are presented in Figure 2b. Here, the reaction rate is expressed in terms of the conversion for each of the phosphodiester linkages in the gap, which is obtained by dividing the total conversion at the gap site by the number of linkages therein. In lanes 4 and 5, a 10-base gap ranging from T40 to C49 was formed by using DNA-L and DNA-R2. In the presence of 1, the gap site was preferentially hydrolyzed (lane 5). This gap-selective DNA scission assisted by 1 is far faster than that without it (lane 4) (22). Synergetic cooperation between the Ce(IV)/EDTA complex and 1 was also observed when a 5-base or 15-base gap was formed in the substrate DNA (lanes 2–3 and 6–7, respectively). The conjugate 1 itself is entirely inactive in the DNA hydrolysis, so that the cooperation of the Ce(IV)/EDTA complex and 1 is evident. The dipropylene-triamine–acridine conjugate 2 and the propylenediamine–acridine conjugate 3 also significantly enhanced the selective DNA scission at the gap site (see text for details).

In contrast with the remarkable acceleration by the conjugates 1–3, quinacrine 4 (an acridine derivative having an alkylated exocyclic amino group; see Fig. 1a) showed no measurable effect on the hydrolysis at the gap site under the conditions employed (lane 4 in Fig. 3b). Spermidine (lane 6), dipropylene-triamine and propylenediamine without acridine.
NH(CH₂)₄NH(CH₂)₃NH₂] was far less active than 1 with lane 3 in Fig. 3b). Apparently, the acridine in it has four positive charges at pH 7.0 as does in terms of the conversion for each of the phosphodiester increasing concentration of hydrolysis at the gap site monotonously increased with 10 linkages in this gap site. In contrast with this significant promotion by 1 of the hydrolysis at the gap site, the hydrolysis of the double-stranded portion is little affected by 1 (open circles). Accordingly, the gap-selective scission becomes clearer with increasing concentration of 1.

For the purpose of comparison, the results for the reactions using spermine (without acridine) are shown in Figure 4b. In the concentration range employed, the hydrolysis of either the gap site (closed triangles) or the double-stranded region (open triangles) is only marginally promoted by spermine. At the higher concentrations of spermine, the rate of hydrolysis at the gap site gradually increased with the increase in its concentration and attained a plateau when its concentration was ~800 μM. In order to obtain an acceleration of a similar magnitude, the concentration of spermine must be more than 10 times as large as that of 1. Strong binding of 1 to the gap site in substrate DNA is primarily responsible for its remarkable activity as a cocatalyst.

Spectroscopic analysis of the binding of 1 to DNA

In order to shed light on the role of the oligoamine–acridine conjugates for the present cooperative catalysis, the binding of 1 to the gap site was quantitatively analyzed. The DNA specimens in Figure 5 were used. DNA-1 has both a single-stranded portion (gap site) and two flanking double-stranded portions. Sufficiently long and flexible oligo(ethylene glycol) linkers are employed to stabilize these two double-stranded portions. DNA-2 and DNA-3 are exactly identical to the non-gap version of DNA-1, and forms a stable duplex throughout the strand.

The binding of 1 to the double-stranded portion. First, the binding of 1 to the double-stranded DNA was investigated. Upon adding double-stranded DNA-4 to an aqueous solution of 1, the visible absorption spectrum of acridine residue in the 400–480 nm region showed a notable hypochromicity. A weak but significant negative sign circular dichroism (CD) was also induced in this region. Furthermore, the fluorescence from the acridine of 1 was strongly quenched by the DNA. All these results show that the acridine residue is intercalating to the DNA. By Scatchard-type analysis of the fluorescence change, the binding constant K and the exclusion number n for the

**Figure 5.** Sequences of DNA oligomers used for the spectroscopic analysis of the binding of 1 to the gap site.

**Figure 4.** (a) Effect of the concentration of 1 on the DNA hydrolysis at the 10-base gap site (closed circles) or in the double-stranded portion (open circles). (b) Effect of the concentration of spermine on the DNA hydrolysis at the 10-base gap site (closed triangles) or in the double-stranded portion (open triangles). The efficiency of scission is expressed in terms of the conversion for each of the phosphodiester linkages, which is obtained by dividing the total conversion at the gap site by the number of linkages.

Kinetic study on the gap-selective DNA hydrolysis by the combination of the Ce(IV)/EDTA complex and 1

By using both DNA-L and DNA-R1, a 10-base gap system was formed. As shown in Figure 4a (closed circles), the rate of hydrolysis at the gap site monotonously increased with increasing concentration of 1. The reaction rate is expressed in terms of the conversion for each of the phosphodiester linkages in the gap, and thus is directly comparable with the corresponding values (open circles) for the double-stranded region in which the number of linkages is different. When [1] = 50 μM and the reaction time was 16 h, the total conversion for the DNA hydrolysis at the gap site was ~15 mol%. Thus, the conversion for each of the linkages was 1.5 mol% (there exist 10 linkages in this gap site). Upon adding double-stranded DNA-4 to an aqueous solution of 1, the visible absorption spectrum of acridine residue in the 400–480 nm region showed a notable hypochromicity. A weak but significant negative sign circular dichroism (CD) was also induced in this region. Furthermore, the fluorescence from the acridine of 1 was strongly quenched by the DNA. All these results show that the acridine residue is intercalating to the DNA. By Scatchard-type analysis of the fluorescence change, the binding constant K and the exclusion number n for the
intercalation were determined to be $4.1 \times 10^6$ M$^{-1}$ and 2.1, respectively (the spectroscopic data and the Scatchard plots are provided in Supplementary Material). This binding constant is approximately 30 times as large as that for the binding of quinacrine to double-stranded DNA ($1.3 \times 10^5$ M$^{-1}$) (25). The binding of 1 to DNA is enhanced by the electrostatic interactions between the positive charges of 1 and the anionic charges of DNA.

The binding of 1 to the gap portion. When DNA-1 involving a 10-base gap was mixed with 1 at pH 7.0, both a hypochromicity in the 400–480 nm region and a weakly induced CD signal were observed (Fig. 6a and b, respectively). These changes are consistent with those in the DNA-4/1 system described above, and correspond to the intercalation of the acridine group of 1 to the double-stranded portions of DNA-1. However, the following analyses definitely substantiate that the conjugate 1 is efficiently bound to both the gap region and the double-stranded portions.

The closed circles in Figure 7a show the plot of the change of absorbance at 421 nm as the function of the concentration of DNA. The open circles refer to the corresponding results for the 1:1 mixture of DNA-2 and DNA-3 (open circles). These plots should superimpose each other. However, the points for the DNA-1 (closed circles) enormously deviate in a positive direction from those for the 1:1 DNA-2/DNA-3 mixture (open circles). Similarly, the intensities of the induced-CD signal for DNA-1 are notably different from the corresponding values for the 1:1 DNA-2/DNA-3 mixture (Fig. 7b). Evidence for the binding of 1 to the gap site is conclusive. Its acridine group should show a stacking interaction with the DNA, whereas the positively charged oligoamine moiety is attracted by the negatively charged DNA. This binding is further promoted by the positive charge on the acridine. These arguments are supported by the fact that the plots for the 1:1 DNA-2/DNA-3 mixture, in both Figure 7a and b, fairly fit the theoretical plots (solid lines) obtained by using the binding constant $K$ and the exclusion number $n$ for the DNA-4/1 system (determined above by the Scatchard analysis).

Proposed mechanism for the gap-selective DNA scission by the combination of the Ce(IV)/EDTA complex and oligoamine–acridine conjugates

The primary reason for the promotion of the scission at the gap site by these conjugates is their efficient binding to the gap site. The apolar interaction of the acridine cooperates with the electrostatic interactions of the oligoamine and the acridine.

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**Figure 6.** (a) Absorption spectra of 1 with (dotted lines) or without (solid lines) DNA-1. (b) CD spectra of DNA-1 with (dotted lines) or without (solid lines) 1. The concentrations of DNA-1 and 1 are 5 and 50 μM, respectively.

**Figure 7.** Changes of (a) the absorbance at 421 nm and (b) CD intensity at 455 nm on the addition of 1 to either DNA-1 (closed circles) or the 1:1 mixture of DNA-2 and DNA-3 (open circles). The solid lines are the theoretical lines calculated by using the binding constant ($4.1 \times 10^6$ M$^{-1}$) and exclusion number ($n = 2.1$) obtained by the Scatchard analysis on the DNA-4/1 system (see text for details). The concentration of 1 is 50 μM in (a) and the concentration of DNA oligomer is 5 μM in (b).
The negatively charged transition state for the DNA hydrolysis is stabilized by the electrostatic interactions with the positive charges of the conjugates (21). These positive charges are greatly accumulated at the gap site, and exhibit notable electrostatic catalysis. In DNA hydrolysis, the negative charges at the reaction center are increased in the transition state, although the net charges of the whole system are unchanged during the reactions. Thus, the positive charges near the reaction center promote the reaction, exactly as does the positive charge of Lys41 of ribonuclease A in the RNA hydrolysis (26). Furthermore, the formation of metal-bound hydroxide near the negatively charged DNA can be assisted by the positive charges. Both of these factors are favorable for DNA hydrolysis. Consistently, spermine (which is not attached to acridine) shows a significant acceleration only when its concentration is sufficiently high (e.g. >500 μM). Quinacrine 4 having two positive charges induces only a marginal effect.

In conclusion, the oligoamine–acridine conjugates are efficiently bound to the gap sites in DNA. Accordingly, the gap-selective DNA hydrolysis by the Ce(IV)/EDTA complex, reported previously (22), is accelerated due to the electrostatic catalysis by their positive charges. These findings should be useful for the preparation of still more effective catalysts for site-selective DNA scission and also for the design of new DNA-binding molecules.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at NAR Online.

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