Targeted Cleavage of Polynucleotides by Complementary Oligonucleotides Covalently Linked to Iron-Porphyrins†

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ABSTRACT: Oligothymidylates covalently linked to iron-porphyrins were synthesized to target the nuclease activity of Fe-porphyrin to complementary polynucleotides. In the presence of oxygen and a reducing agent, oligo(dT)$_7$ bearing the reactive group attached to the 3'-phosphate was shown to be active in the cleavage of poly(dA) and poly(rA) but not poly(dT). When poly(dA) was used as a matrix, the reaction yield was higher at low temperature where the complexes are stable; upon increasing temperature, the reaction yield decreased in agreement with the dissociation of the oligonucleotide-polynucleotide complex as measured by absorption spectroscopy. Thus, oligonucleotides covalently linked to iron-porphyrin derivatives can be used to cleave selectively the target sequence of the oligonucleotide.

Site-directed chemical modification of nucleic acids has been the subject of active research by several groups during the past 10 years. The rationale followed by researchers engaged in this field is based on the idea of associating a recognition group and an active reagent. The recognition part of such hybrid molecules includes compounds that interact strongly with DNA, e.g., intercalators, or exhibit binding specificity for some topological features of the double helix (major vs. minor groove, left-handed region, etc.), or possess sequence specificity (AT- vs. GC-rich regions, complementary oligonucleotide sequences). Reactive groups are compounds that can achieve chemical modification of the nucleic acid bases (Grineva et al., 1977; Vlassov et al., 1983) or cleave the phosphodiester backbone (Boutorin et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Boidot-Forget et al., 1986; C. H. B. Chen and D. S. Sigman, personal communication). Nuclease activity is a characteristic feature of several metal-chelating substances such as ethylenediaminetetraacetic acid (EDTA)[1] or 1,10-phenanthroline. In the presence of oxygen and a reducing agent, redox reactions take place producing oxy radicals that cleave the phosphate-sugar chain. Oligonucleotides of defined sequence coupled to active groups such as EDTA-Fe(II) (Boutorin et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Boidot-Forget et al., 1986) or 1,10-phenanthroline-Cu(I) (Chen and Sigman, personal communication) have been shown to be efficient in the cleavage of complementary nucleic acid sequences in the presence of a reducing agent in aerated solutions.

In this paper, we present results on the cleavage of poly(dA) and poly(rA) by complementary oligo(dT) linked to iron-porphyrins. The reaction was highly specific as cleavage occurred only with complementary matrices and in a temperature range where hybrids were formed.

Materials and Methods

Porphyrin Metalation. Metalation of oligothymidylate-porphyrin adducts was carried out by heating (60-80 °C) under argon atmosphere an aqueous solution of the porphyrin conjugate in the presence of $FeCl_2$ at a Fe:porphyrin ratio ranging from 20 to 50. The reaction was followed by absorption spectroscopy: the sharp Soret band at 400 nm was converted to a broader band centered at 390 nm; the four visible bands (460-650 nm) collapsed into two bands when iron-porphyrin reached its final state as Fe(III)-porphyrin. The reaction was found to be completed after heating for 30-90 min, depending on the type of derivative and the Fe:porphyrin ratio. Excess iron was removed by treating the reaction mixture with ion-exchange resins (Chelex 100 or Dowex 50; Bio-Rad). Colorimetric titration of residual iron was carried out by complexing Fe^{2+} with *o*-phenanthroline. Residual iron in the porphyrin solution was always kept below 5×10^{-7} M.

Labeled Polynucleotides. Poly(rA), poly(dA), and poly(dT) were purchased from P-L Biochemicals. 5'-^{32}P-Labeled polynucleotides resulted from dephosphorylation of the corresponding polynucleotides with bacterial alkaline phosphatase (BRL) and phosphorylation with [γ-^{32}P]dATP and T_4 polynucleotide kinase (Amersham). 3'-^{32}P labeling was carried out with [α-^{32}P]dATP and nucleotidyl terminal transferase (Amersham). To obtain a homogeneous length distribution of poly(rA), the polymer was first loaded on an 8% polyacrylamide gel and electrophoresed for 2 h under a voltage of 1200 V. The gel was then autoradiographed, and the upper part of the gel containing the high molecular weight fraction (≈1-cm width) was sliced out. The gel bands were then slipped in dialysis bags and submitted to electrodiffusion. The polymer was recovered from the buffer solution, precipitated, washed, and lyophilized. All experiments were carried out with sterile solutions in equipment treated by 0.1 M NaOH solutions to inactivate traces of ribonuclease.

Cleavage Reactions. Cleavage reactions were performed in Eppendorf tubes containing buffered solutions (10 mM

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; MPPo, methylpyrroporphyrin; Acr, 2-methoxy-6-chloro-9-aminoacridine; T_7PORFe(1), T_7PORFe(2), and PORFeT_7Acr, iron derivatives of **1**, **2**, and **3**, respectively (see Scheme I); HPLC, high-pressure liquid chromatography.

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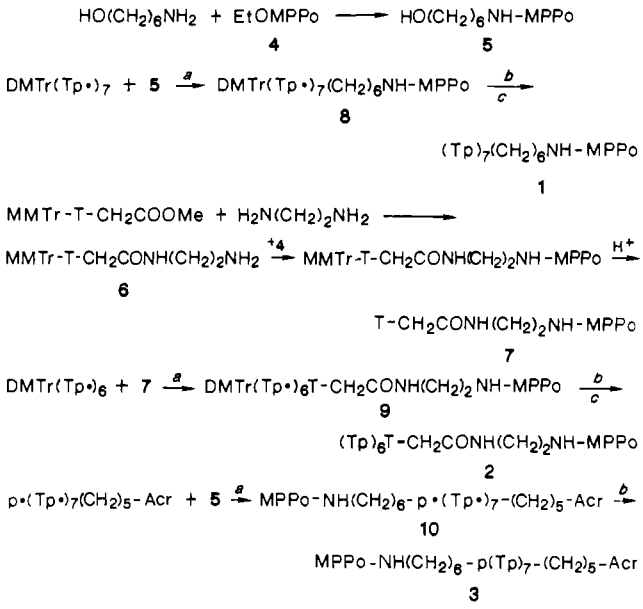
Scheme I: Synthesis Scheme of Oligothymidylates Covalently Linked to MPPo and to MPPo and Acridine Derivatives



[a] Mesitylenesulfonyl tetrazolide. [b] Benzohydroxamic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene. [c] Acetic acid; Me, methyl; Et, ethyl.

sodium phosphate, pH 7.4, and 150 mM NaCl) of the polynucleotide and the porphyrin derivative. The mixture was then incubated at the appropriate temperature, and dithiothreitol (DTT) (Sigma) was added to start the cleavage reaction. At a definite time, tubes were removed, quenched in a dry ice bath for 5 min, and lyophilized. The polynucleotide was redissolved in 4 μL of formamide and immediately loaded on an 8% polyacrylamide [acrylamide:bis(acrylamide) ratio of 1:20]–urea (7 M) gel, 0.5-mm thick. Electrophoresis was usually run for 2–3 h under 1000–1300 V. Autoradiography was carried out by using X-ray Fuji film at −80 °C with an intensifying screen. The extent of cleavage was determined by comparing the radioactivity of the intact polymer to that of the degraded polymer by counting the corresponding bands excised from the gel.

Results and Discussion

Synthesis of the Oligonucleotide–Porphyrin Adducts. Two oligothymidylates (**1** and **2**) containing a porphyrin derivative attached at their 3′ end and one (**3**) containing both an acridine attached at the 3′ end and a porphyrin at the 5′ end were synthesized according to Scheme I. Full details of the synthesis will be given elsewhere (N. T. Thuong et al., unpublished results). In short, methylpyrroporphyrin XXI ethyl ester (MPPo) was reacted with amino-1-hexanol and then with the protected oligonucleotide DMTr(Tp•)$_7$ (where DMTr is dimethoxytrityl and p• is a *p*-chlorophenyl phosphoester). After deprotection and HPLC purification, compound **1** was obtained.

Alternatively, MPPo was reacted with a thymidine derivative whose 5′-OH was substituted by a monomethoxytrityl group (MMTr) and 3′-OH by $CH_2CONH(CH_2)_2NH_2$ (**6**). This thymidine derivative was obtained by reaction of ethylenediamine with the 3′-acetyl methyl ester derivative. The nucleotide sequence was then extended by coupling the detritylated product with DMTr(Tp•)$_6$ to give compound **2** after deprotection and purification by HPLC.

The oligonucleotide **3** containing an acridine derivative (Acr) attached at the 3′ end via a pentamethylene linker and MPPo

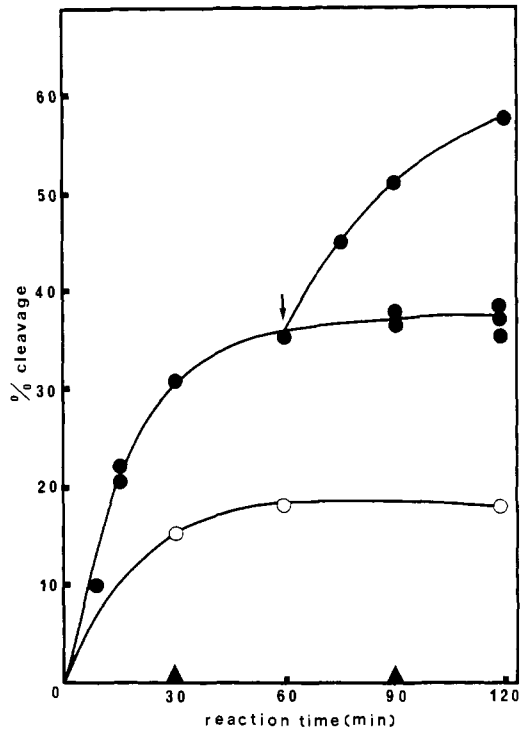


FIGURE 1: Kinetics of cleavage of poly(dA) (●), poly(rA) (○), and poly(dT) (▲) by T_7PORFe(2) in the presence of 5 mM DTT at 0 °C in aerated solutions (10 mM sodium phosphate), pH 7.4, and 150 mM NaCl). [Polynucleotides] = 45 μM; [T_7PORFe] = 140 μM (in nucleotide units, i.e., 20 μM in PORFe). The arrow indicates the addition of an equivalent quantity of T_7PORFe after 1-h reaction.

attached at the 5′-phosphate via a hexamethylene linker was synthesized by condensation of the ω-hydroxyl of **5** with the 5′-phosphodiester of p•(Tp•)$_7$–$(CH_2)_5$–Acr. The latter was obtained as previously described (Asseline et al., 1986). After dearylation, compound **3** was purified by HPLC.

The corresponding iron derivatives of **1**, **2**, and **3** used in this work will be named T_7PORFe(1), T_7PORFe(2), and PORFeT_7Acr, respectively.

Porphyrin complexes of Fe(III), Mn(III), and Co(III) were previously shown to induce cleavage of polynucleotide chains in the presence of reducing agents such as thiol derivatives or ascorbic acid (Ward et al., 1985). Covalent linkage of the porphyrin derivative to an oligonucleotide was expected to bring the cleaving reagent in close proximity to the complementary sequence and therefore to confer upon this molecule a targeted nuclease activity. The additional attachment of an acridine ring to the oligonucleotide was expected to stabilize the hybrid (Asseline et al., 1984).

The time course of cleavage of poly(dA) and poly(rA) by T_7PORFe(2) in the presence of 5 mM DTT at 0 °C is presented in Figure 1. The extent of reaction increased with time and reached a plateau after about 1-h incubation. Under the concentration conditions indicated under Figure 1, the reaction leveled off at 37% for poly(dA) and at 20% for poly(rA). Under the same conditions, poly(dT) and poly(dC) were not cleaved, indicating that the cleavage reaction was specific of the complementary sequence of the oligonucleotide (Figure 1). Hydrolysis of poly(dA) was not observed in the absence of DTT. The oligonucleotide–porphyrin had no effect when the metal was not incorporated into the porphyrin ring. Similar results were observed when T_7PORFe(1) was used instead of T_7PORFe(2) to cleave poly(dA). The porphyrin ring chosen in our experiments did not bear any positive charge in order to avoid nonspecific electrostatic binding to any negatively charged nucleic acid. When it was not tethered to

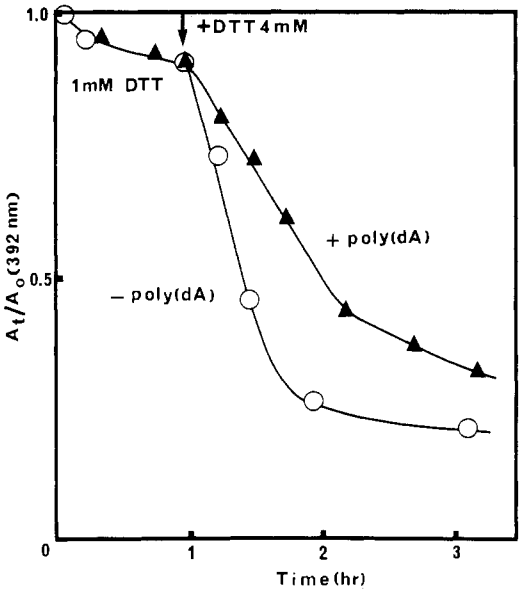


FIGURE 2: Chemical modification of the porphyrin ring of T_7PORFe(1) in the presence of DTT at 7 °C and in the presence (▲) and absence (○) of poly(dA). The concentrations are identical with those indicated under Figure 1 except for DTT whose concentration was 1 mM at the beginning of the reaction and then increased to 5 mM after 1-h incubation. A_0 and A_t are the absorbances of the Soret band at 0 and t hours incubation, respectively, after addition of DTT.

an oligo(dT), it did not bind to any polynucleotide and did not induce any cleavage of single and double strands under the same concentration conditions. The nonspecific cleavage observed at higher temperatures (see below) indicated that the discrimination between poly(A) and other polynucleotides at 0 °C was not due to a sequence preference in cutting by the porphyrin but did result from the targeting of the porphyrin ring to the complementary sequence of the oligonucleotide.

The plateau observed after 1-h incubation under the conditions of Figure 1 could be due to a degradation of the oligonucleotide–porphyrin derivative during the reaction. In agreement with this hypothesis, the cleavage reaction of poly(dA) resumed when a fresh solution of T_7PORFe(2) was added to the reaction mixture after 1-h incubation (Figure 1). Iron derivatives of **1** and **2** are stable in aqueous solution in the absence of DTT. When ^{32}P labeled at their 5′ end by polynucleotide kinase, these modified oligonucleotides migrated as a single band on a denaturing 20% polyacrylamide–7 M urea gel. As expected, the porphyrin ring retarded the migration as compared to the free oligonucleotide (results not shown). After incubation in the presence of 5 mM DTT, shorter fragments were not detected, indicating that the oligonucleotide chain was not cleaved. However, evidence for a chemical modification of the porphyrin moiety was provided by absorption spectroscopy with a strong reduction of the Soret band at ≈392 nm (Figure 2). The presence of an isosbestic point at 330 nm indicated that the porphyrin ring was converted to a single (unidentified) compound. No cleavage of the linker between the porphyrin ring and the oligonucleotide was observed. Such a cleavage would have changed the mobility of the ^{32}P-labeled oligonucleotide on polyacrylamide gels, which was not detected. In the presence of poly(dA), the chemical modification of the porphyrin ring was slowed down, indicating that hybrid formation between the oligo(dT) and its complementary sequence partially protected the porphyrin from self-modification as shown on Figure 2. The rate of porphyrin modification strongly depended on DTT concentration. At 1 mM DTT, little change occurred in the absorption spectrum. Yet the cleavage of poly(dA) was still

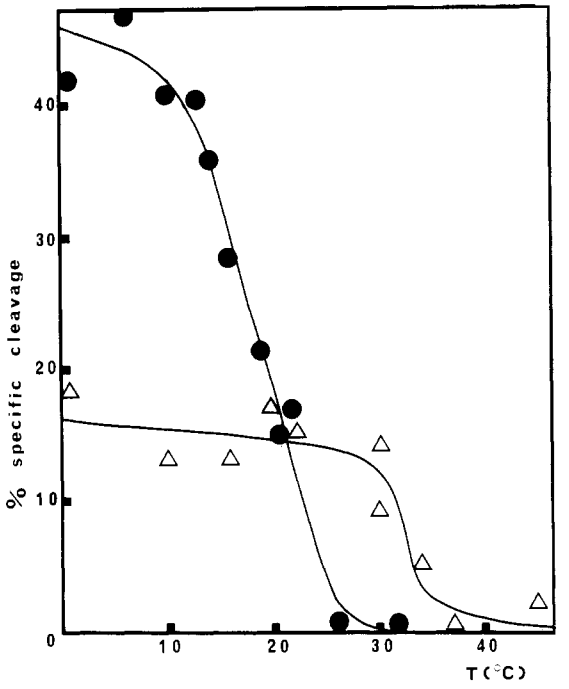


FIGURE 3: Specific cleavage of poly(dA) by T_7PORFe(1) (●) and PORFeT_7Acr (△) as a function of temperature. Specific cleavage was measured by subtracting from the observed yield that measured on a poly(dT) matrix (nonspecific cleavage) under identical conditions of concentration and temperature. The concentrations of poly(dA), oligonucleotide, and DTT are the same as those indicated under Figure 1.

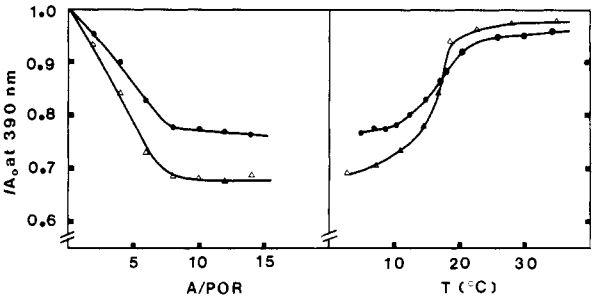


FIGURE 4: (Left) Titration of T_7PORFe(1) by poly(dA) (●) and poly(rA) (△) at 0 °C. The concentration of T_7PORFe was 1.5 μM in porphyrin. A/POR = adenine/porphyrin. (Right) Melting profiles for the above complexes. The observed T_m (temperature at half-transition) for the poly(dA) complex was 17 °C and for the poly(rA) complex 16 °C.

observed with the same efficiency as in the presence of 5 mM DTT. Therefore, porphyrin modification was a secondary reaction involving the thiol derivative which can be avoided by using low DTT concentrations.

The reaction rate for the cleavage of poly(dA) by T_7PORFe(1 and 2) remained constant between 0 and about 10 °C. Above 10 °C, the rate dropped rapidly (Figure 3). Above 20 °C, a nonspecific reaction of T_7PORFe(2) with poly(dT) was detected whose rate increased with temperature as already observed for the EDTA–Fe(II) cleavage reaction (Boidot-Forget et al., 1986). At 30 °C, the rate of nonspecific cleavage was about 10% that of the targeted cleavage observed at 0 °C for poly(dA). The decrease in the reaction rate observed above 10 °C in the case of poly(dA) reflects the dissociation of the oligonucleotide from its polynucleotide matrix as measured from the temperature dependence of the absorption spectrum (Figure 4). Binding of T_7PORFe(1) to poly(dA) and poly(rA) at 0 °C induced a hypochromism in the Soret absorption band. The stoichiometry of the reaction

was in agreement with the formation of a 1:1 A–T complex. Dissociation of the poly(dA) complex was observed with a half-transition temperature of 17 °C, in good agreement with the temperature of 19 °C determined from the temperature dependence of the cleavage reaction. It should be noted that the cleavage reaction was carried out at a higher concentration of oligonucleotide than the spectroscopic studies which explains the observed difference in melting temperature. These results demonstrated that the cleavage reaction at 0 °C was due to the bound oligonucleotide and that dissociation abolished specific cleavage.

The efficiency of cleavage depended on the length of the linker used to attach the porphyrin derivative to the 3′ end of the oligonucleotide, the shorter linker being less efficient (results not shown). The oligonucleotide covalently linked to an acridine derivative at the 3′ end and to the porphyrin ring at its 5′ end ($PORFeT_7Acr$) was less efficient at low temperature than T_7PORFe(1 and 2), but specific cleavage was observed at higher temperatures when the acridine was present, in agreement with hybrid stabilization afforded by the intercalating acridine ring (Asseline et al., 1984). The lower activity at 0 °C might be due to the lower efficiency of cleavage when the porphyrin ring is attached at the 5′ rather than the 3′ end. Similar behavior was previously observed with the EDTA–Fe complex (Boidot-Forget et al., 1986; unpublished results). Due to cooperative binding of oligo(dT)'s along poly(dA), bis substitution of the oligonucleotide brings the porphyrin ring of one oligonucleotide close to the acridine group of the following one. This intermolecular interaction might also be responsible for the lower observed cleavage efficiency. Studies with an isolated target sequence should help clarify this point.

Conclusions

The results presented in this study demonstrate that oligonucleotides covalently linked to iron–porphyrins induce a cleavage reaction of complementary polynucleotides in the presence of a reducing agent. It was previously shown in our laboratory that oligonucleotides covalently linked to intercalating agents such as acridine derivatives could specifically block biological processes such as transcription and translation both in vitro and in vivo (Hélène et al., 1985; Toulmé et al., 1986). In this family of modified oligonucleotides, the role of the acridine derivative was to increase the stability of the hybrids (Asseline et al., 1984). Even though the intercalating agent increases the residence time of the oligonucleotide on its target, it might be helpful to increase the efficiency of the biological effect by producing an irreversible chemical lesion in the target. The induced damage is expected to block elongation processes during replication, transcription, or translation. Site-directed cleavage by metal–porphyrin derivatives covalently linked to an oligonucleotide represents an extreme case of such irreversible reactions where the target sequence is chemically cleaved by radical intermediates. Oligonucleotide–porphyrin derivatives are promising for the development of new "anti-gene" or "anti-messenger" molecules.

Acknowledgments

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