Enzymatic transfer of ATPγS thiophosphate onto the 5′-hydroxyl of an oligonucleotide as a route to reactive oligonucleotide derivatives

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Received 8 April 1982

Polynucleotide kinase *Thiophosphorylation* *Oligonucleotide derivatives*

1. INTRODUCTION

Thiophosphate analogs of NTP-S, obtained for the first time in [1,2], are now widely used for studies of the mechanisms of enzymatic reactions [2,3] (other applications [4–6]). Of greatest interest is adenosine 5′-*O*-(3-thiotriphosphate) because it acts as a substrate of phosphorylating enzymes [2].

This investigation showed that polynucleotide kinase of T4 phage transfers the thiophosphate residue of ATPγS onto the 5′-hydroxyl of the deoxy-oligonucleotide d-CpTpTpTpCpCpA. Alkylation of this thiophosphorylated oligonucleotide by polyfunctional reagents has also been studied; the high reactivity of the thiophosphate group in alkylation gives a convenient route to reactive derivatives of oligonucleotides, which may be used for affinity modification of proteins and nucleic acids.

2. MATERIALS AND METHODS

Adenosine 5′-*O*-(3-thiotriphosphate) was purchased from Serva (Heidelberg); alkaline phosphatase BAPF from Millipore (London); Sephadex G-25 from Pharmacia (Uppsala); ATP and HEPES from Merck (Darmstadt). *N,N,N′*-Tris-(2-chloroethyl),*N′*-(*p*-formylphenyl) propylenediamine 1,3 (Cl_3R) [7] was kindly given by Dr Gall; *N*-(2-chloroethyl), *N*-methyl, *N*-*p*-azidobenzylamine ($N_3R'Cl$) by Dr Dobrikov (Novosibirsk Institute of Organic Chemistry). The absorbent 'Aminosilochrom' the properties of which are close to that of Lichrosorb-NH_2, and T4 polynucleotide kinase were kindly given by Drs Kumarev and Rivkin of this Institute. The oligonucleotide d-CpTpTpTpCpCpA was synthesized by Dr Sinyakov using the procedures of Narang.

Analysis of reaction mixtures by micro-column liquid chromatography combined with multiwavelength detection was done as in [8] using the liquid chromatograph Ob-4.

2.1. *Transfer of thiophosphate from ATPγS onto the oligonucleotide d-CpTpTpTpCpCpA*

The reaction was run with T4 polynucleotide kinase under conditions similar to those employed with ATP [9]: 0.05 M Tris–HCl pH 7.6 (9.5)–0.01 M $MgCl_2$–3 mM DTT–0.1 mM EDTA, 0.5 mM ATPγS, 0.07–0.15 mM d-CpTpTpTpCpCpA; 200 activity units/ml polynucleotide kinase. This mixture was incubated at 37°C. At time intervals aliquots were analyzed by micro-column liquid chromatography (fig.1). In 40 h of reaction, the product was isolated by chromatography on 'Aminosilochrom'. The fractions of the product were combined and, in order to change the buffer, subjected to gel-filtration on Sephadex G-25 in 5 mM Hepes (pH 7.3)–0.1 mM EDTA or 0.02 M H_3BO_3–NaOH (pH 8.3)–1 mM EDTA. The 5′-thiophosphorylated oligonucleotide was concentrated to 30 μM by evaporation and stored at – 20°C.

2.2. *Dephosphorylation by* E.coli *alkaline phosphatase*

Oligonucleotide (0.5 nmol) in 20 μl 0.06 M H_3BO_3–NaOH pH 8.3–3 mM EDTA were incubated with 0.02 activity units of phosphatase during 1 h at 37°C, and analyzed by micro-column liquid chromatography. The thiophosphorylated oligo-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

nucleotide did not change its chromatographic mobility, whereas the same oligonucleotide with a phosphate group at the 5'-terminus was completely dephosphorylated, as revealed by an increase of the chromatographic mobility expected for a product lacking two negative charges.

2.3. *Synthesis of the azido-derivative of the thiophosphorylated oligonucleotide*

The reaction mixture contained 25 μM thiophosphorylated oligonucleotide–300 μM *N*-(2-chloroethyl),*N*-methyl,*N*-*p*-azidobenzylamine and buffer (0.16 M H_3BO_3–NaOH (pH 8.3)–8 mM EDTA or 0.07 M Hepes (pH 7.3)–1.4 mM EDTA). The reaction was for 3 h at 20°C. In control experiments alkylated was phosphorylated oligonucleotide d-pCpTpTpTpCpCpA. The reaction was run for 1 h at 50°C. The results of the analysis of reaction mixtures by micro-column liquid chromatography are shown in fig.4.

2.4. *Alkylation of the thiophosphorylated oligonucleotide by Cl_3R*

Thiophosphorylated oligonucleotide (30 μM) in 0.02 M Hepes (pH 7.3)–0.4 mM EDTA was mixed with a concentrated solution of Cl_3R in dimethylformamide to give a 200 μM alkylating agent, and the mixture incubated for 1 h at 20°C. The reaction mixture was analyzed by micro-column liquid chromatography. In control experiments, the phosphorylated oligonucleotide was alkylated; however, no reaction was detected after incubation under the above conditions; hence it was run with 400 μM Cl_3R for 1 h at 40°C (fig.3).

3. RESULTS

3.1. *Thiophosphorylation*

It was found that 40 h incubation of the oligonucleotide d-CpTpTpTpCpCpA with excess ATPγS and T4 phage polynucleotide kinase resulted in accumulation of a product which elutes on 'Aminosilochrom' chromatography much later than the starting oligonucleotide and slightly earlier than ATPγS *; this product had the same spectral characteristics as those of the starting oligonucleotide. The peak of the starting oligonucleotide (→) disappeared almost completely. No changes of the reaction mixture were detected by chromatography in control experiments in the absence of polynucleotide kinase. At shorter incubation times both at pH 7.6 and pH 9.5 the enzymic reaction did not go to completion.

The product which was eluted before ATPγS (fig.1, peak 2) was tentatively identified as the thiophosphorylated oligonucleotide *S*-dpCpTpTpTpCpCpA and this conclusion was confirmed by the data discussed below:

(1) Its chromatographic mobility was slightly lower than that of the phosphorylated oligonucleotide d-pCpTpTpTpCpCpA (fig.2); similar difference of mobilities is characteristic of ATPγS and ATP [6].

(2) The product is stable to *E.coli* alkaline phosphatase; this property is also in line with the fact that ATPγS, unlike ATP, is not dephosphorylated by the enzyme.

(3) The presence of a thiophosphate residue was confirmed by the chemical reactivity of *S*-dpCpTpTpTpCpCpA (section 3.2).

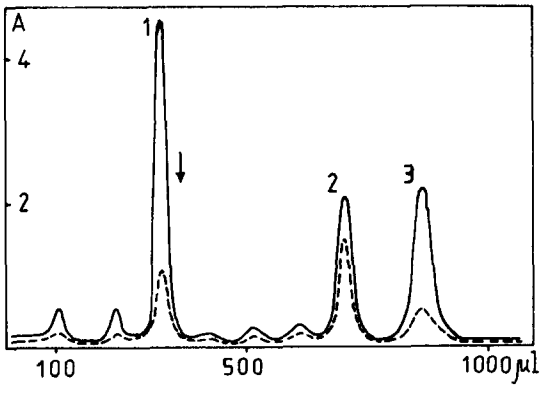
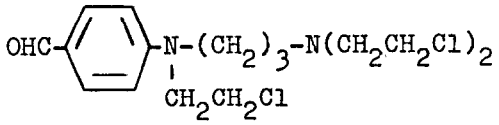


Fig.1. Micro-column chromatography of the reaction mixture of ATPγS, d-CpTpTpTpCpCpA and polynucleotide kinase after 40 h incubation (section 2.1). Conditions of chromatography: 70 μl 'Aminosilochrom' column, linear gradient of potassium phosphate (pH 7.5) from 0–0.11 M (1.2 ml) in 7 M urea; rate 50 μl/min); (—) A_{260}; (- - -) A_{280}; (1) ADP; (2) *S*-dpCpTpTpTpCpCpA; (3) ATPγS; (→) position of d-CpTpTpTpCpCpA.

* 'Aminosilochrom' has specific affinity to polyphosphate residues and hence polyphosphates like ATP or ATPγS are eluted much later, than could be expected on the basis of their net charge

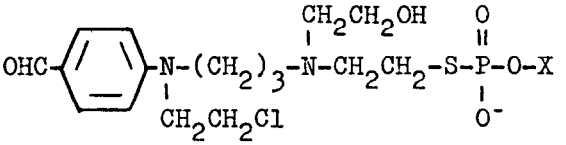
3.2. *Alkylation of the 5'-thiophosphorylated oligonucleotide*

In [10] thiophosphorylated dinucleotide *S*-dpTpT was reacted with ethyl bromide in 50% pyridine to obtain its *S*-ethyl derivative [10]. We alkylated our thiophosphorylated oligonucleotide with the trifunctional alkylating reagent Cl_3R [7]:



The two 2-chloroethyl groups of this reagent, which reside at an atom with an aliphatic substituent are highly reactive. The third 2-chloroethyl group at the nitrogen atom with the *p*-formylphenyl residue is inactive due to the electron-acceptor effect of the formyl group, but may be activated by means of reduction of the formyl residue with sodium borohydride. This compound was used to introduce a 'latent' alkylating group into RNA in [11] and also into ATP by means of alkylation of its γ-phosphate residue [12]. The formylphenyl residue of Cl_3R strongly absorbs at 350 nm, and it is convenient to look for the products of alkylation by Cl_3R using this absorption band.

The reaction of Cl_3R with *S*-dpCpTpTpTpC-pCpA under mild conditions results in its complete alkylation at the 5'-thiophosphate residue, as revealed by micro-column liquid chromatography with multiwavelength detection (fig.3). The product obtained exhibits absorbance at 350 nm, and the A_{350}/A_{260} ratio (cf. [12]) suggests that 1 formylphenyl residue/mol oligonucleotide is present. The chromatographic mobility suggests a decrease of the net negative charge. By analogy with the product of reaction of Cl_3R with ATP [12], it is assumed that the 5'-thiophosphate group of the oligonucleotide derivative obtained is substituted in the following way:



X - oligonucleotide

The efficiency of the alkylation of the thiophosphorylated oligonucleotide is much greater than that of the phosphorylated one (fig.3).

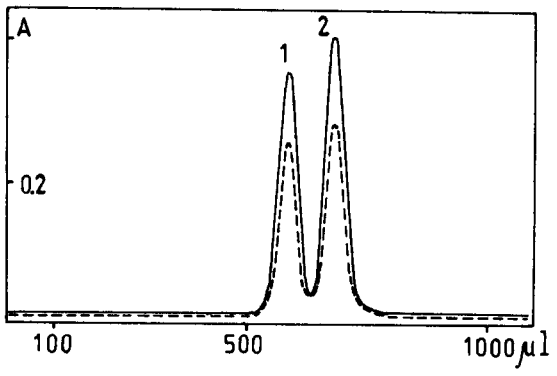


Fig.2. Separation of d-CpTpTpTpCpCpA (1) and S-dpCpTpTpTpCpCpA (2); conditions as in fig.1; (——) A_{260}; (- - -) A_{280}.

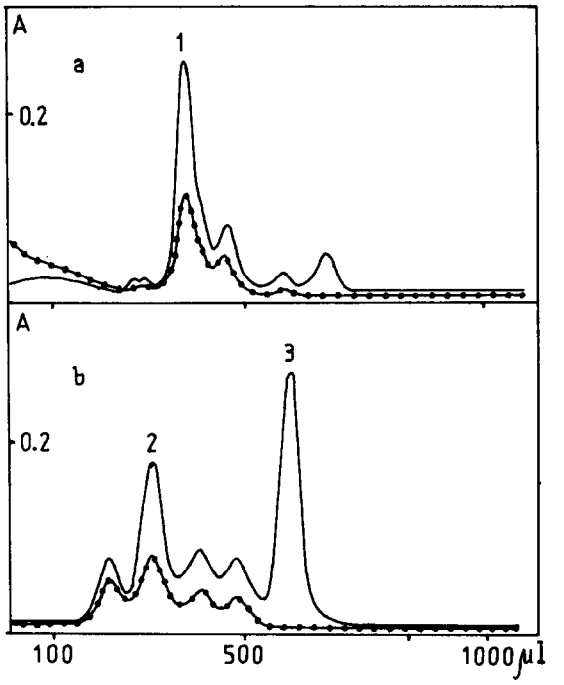
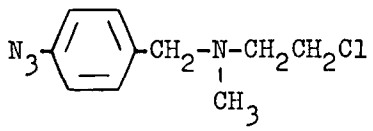


Fig.3. Analysis of the products of reaction of Cl_3R with the thiophosphorylated oligonucleotide (a) and with the phosphorylated oligonucleotide (b): (1,2) products of alkylation of *S*-dpCpTpTpTpCpCpA and d-dpCpTpT-pTpCpCpA, respectively; (3) d-pCpTpTpTpCpCpA; (——) A_{260}; (•••) A_{350}; conditions of chromatography as in fig.1.

The oligonucleotide *S*-dpCpTpTpTpCpCpA was alkylated also by *N*-(2-chloroethyl),*N*-methyl,-*N*-*p*-azidobenzylamine:



to obtain a photo-reactive derivative. The reaction of $N_3R'Cl$ with *S*-dpCpTpTpTpCpCpA results in its complete alkylation at the 5′-thiophosphate residue, as revealed by micro-column liquid chromatography (fig.4a). The efficiency of alkylation of the thiophosphorylated oligonucleotide is much greater than that of the phosphorylated one (fig.4).

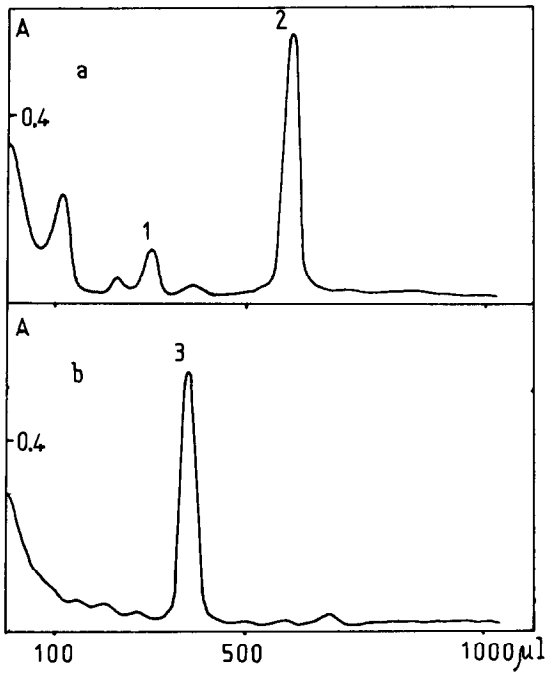


Fig.4. Analysis of the products of alkylation of d-pCpTpTpTpCpCpA (a) and S-dpCpTpTpTpCpCpA (b) with $N_3R'Cl$: (1) the product of alkylation of the phosphorylated oligonucleotide; (2) the starting phosphorylated oligonucleotide; (3) the product of alkylation of the thiophosphorylated oligonucleotide; (———) A_{260}; conditions of chromatography as in fig.1.

4. DISCUSSION

To evaluate the selectivity of the method of derivatization of oligonucleotides proposed, one may compare the competition factors of thiophosphate ($\sim 2 \times 10^6$), phosphate (10^4) and nucleic acid base residues ($\sim 3 \times 10^3$) in alkylation with aliphatic 2-chloroethylamines [13]. The reactivity of the thiophosphate group is ≥ 3 orders of magnitude greater than that of normal nucleic acid components. Hence, the approach proposed gives such a high selectivity, which could not be achieved by other techniques [14,15].

Using this approach many different types of reactive and reporter groups may be attached to the 5′-termini of oligonucleotides and nucleic acids. Such reactive derivatives may find numerous applications, e.g., for 'complementary-addressed' chemical modification of nucleic acids [16]; for affinity modification of enzymes, which interact specifically with different oligonucleotide sequences, like restriction nucleases; and for detection and isolation of particular sites of genomes.

When this paper was being prepared for publication, a communication which describes thiophosphorylation of the dinucleotide ApA by T4 polynucleotide kinase and ATPγS appeared [17].

ACKNOWLEDGEMENTS

I am thankful to Drs G.V. Shishkin, A.A. Gall and M. Dobrikov for alkylating amines, to Dr A. Sinyakov for the oligonucleotide, to Drs M.I. Rivkin and V.P. Kumarev for polynucleotide kinase and 'Aminosilochrom' and for interest in this work, to Dr V.S. Bogachev and Professor D.G. Knorre for encouragement and especially to Dr M.A. Grachev for useful critical discussions.

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