AN ENZYMATIC SYNTHESIS OF 5’-END SUBSTITUTED OLIGONUCLEOTIDES
USING T4 POLYNUCLEOTIDE KINASE AND γ-AMIDES OF ATP,
BEARING PHOTOREACTIVE GROUPS

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Key words: T4 polynucleotide kinase; photoreactive ATP derivatives; 5’-end substituted photoreactive oligonucleotides, photoaffinity labelling

Resume

Photoreactive derivatives of oligonucleotides containing arylazido groups at 5’-phosphate introduced by chemical synthesis extensively are used for the investigation of the components of DNA replication/repair systems by the methods of photoaffinity modification. We proposed that ATP derivatives bearing photoreactive substitutions at γ-phosphate might turn out substrates for T4 polynucleotide kinase (T4 PNK) and this allows introducing different groups into the 5’-OH end of oligonucleotides. A set of γ-phosphoamides of ATP was synthesized and shown to be substrates of this enzyme. Photoreactive oligonucleotide conjugate obtained by using of the γ-N-[-(4-azido-2,3,5,6-tetrafluorobenzoyl)-aminoethyl]amide ATP and 17-mer deoxyribooligonucleotide as substrates has been used for the photoaffinity labelling of human replication protein A (RPA) and flap endonuclease 1 (FEN 1). It can be proved that tested set of the γ-amides of ATP can be applied for the realization of the new enzymatic approach synthesis of 5’-end-phosphate substituted derivatives of ribo- and deoxyribooligonucleotides.

Introduction

It was shown for some enzymes of nucleic-acid-metabolism that substitution at γ-phosphate of don't (NTP) didn't suppress substrate properties of nucleoside triphosphate (Grachev et al., 1980; Arzumanov et al., 1996) Adenosine-5’-O-(3-thiophosphate) was successfully used for thiophosphorylation of 5’-OH-group of oligonucleotide using T4 PNK (Oshevski, 1982). We proposed that ATP derivatives bearing other substitutions at γ-phosphate might also turn out substrates for this enzyme. Our interest in such a tool is based on necessity to have a wide range of 5’-end modified oligonucleotides for investigation of the interaction of enzymes and factors of DNA replication and DNA repair with the targeted DNA structures.

Results and Discussion

γ-Phosphamides of ATP I-IV (Fig. 1) were shown to be substrates of T4 PNK.

A pattern of transfer of phosphoryl groups with different substitutions to 14-mer ribooligonucleotide is shown in Fig. 2. The choice of the concentrations of ATP derivatives and oligonucleotides for preliminary trials of new compounds in the T4 PNK catalyzed reaction is based on the known Km values: 65 µM for ATP and 6.5 µM for 5’-OH-polynucleotide substrate (Sano, 1976; Nicols et al., 1978). The efficiency of transfer was shown to depend on the nature of modifying group and required different concentration of ATP analogs to achieve comparable levels of transfer.
Panel A of Fig. 3 represents an example of chromatographic separation the reaction products on the column using multiwave detection and Panel B demonstrates the dependence of phosphorylation level of ON12 on the concentration γ-NAB-ATP.

Efficient transfer of modified phosphoryl groups requires higher concentrations of T4PNK than conventionally used with ATP. Bulky substituents at phosphate group may influence both $K_m$ and $V_{max}$ values. The radioactive photoreactive 5'-FAB-p-derivative of 17-mer oligonucleotide ON17 was used (in the form of duplex) as reagent for photoaffinity modification of two proteins involved in the DNA metabolism, human RPA and FEN 1. The fact of photoinduced labelling of the proteins by enzymatically synthesized 5'-derivative of oligonucleotide (Fig. 4) confirms the transfer of modified phosphoryl group and the integrity of photoreactive arylazido group after the purification procedure used. The difference in apparent molecular masses of the proteins products of their modification is about 8 kDa that corresponds the molecular mass of crosslinked oligonucleotide. RPA, the nuclear single-stranded DNA binding protein, is involved in DNA replication, nucleotide excision repair (NER) and homologous recombination. It is a stable heterotrimer consisting of subunits with molecular masses of 70, 32 and 14 kDa (p70, p32 and p14, respectively (Wold, 1997; Iftode et al., 1999). Using for affinity modification Of RPA realized in the labelling only subunit of the heterotrimer protein RPA (Fig. 4, lanes 1-3) lake the cases when chemically synthesized 5'-photoreactive oligonucleotides were used (Kolpashchikov et al., 2001; Khlimankov et al., 2001a) The higher the concentration of RPA in the reaction mixture the more intensive labelling was (compare lanes 1-3, Fig. 4). In contrast to pattern of RPA labelling the amount of FEN 1 modification product practically was not increased at higher FEN 1 concentrations (compare lanes 4-6, Fig. 4). The peculiarity of FEN 1 labelling appears to reflect faster removing the photoreactive nucleotide from 5'-end of the reagent via exonucleolytical degradation (Lieber, 1997) under the

Fig. 2. Substrate properties of γ-amides of ATP in phosphorylation of rON14 catalyzed by T4 PNK. All reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$ (buff. A), and 15 µM rON14. T4 PNK 2 U/µL. Lanes 1, 2, 3 contained 194 µM, 772 µM, 1544 µM γ-EDA-ATP; lanes 4, 5, 6 - 90 µM, 180 µM, 900 µM γ-FAB-ATP; lane 7 - 75 µM ATP; lane 8 - without NTP; lanes 9, 10, 11 - 33.3 µM, 166 µM and 332 µM γ-FABO-ATP. p*-rON14 designates phosphorylation product of rON14, which corresponds to the phosphoryl residue donor has been used. Reaction mixtures were incubated at 37°C for 30 min and analyzed by PAG electrophoresis under denaturing conditions followed by staining with "Stains all".

Panel A of Fig. 3 represents an example of chromatographic separation the reaction products on the column using multiwave detection and Panel B demonstrates the dependence of phosphorylation level of ON12 on the concentration γ-NAB-ATP.

Fig. 3. Analysis of the T4 PNK-dependent phosphorylation product yield as a function of concentration of γ-NAB-ATP. (A) MLC separation of the reaction mixture containing T4PNK - 1.0 U/µL, 15 µM ON12, 0.3 mM γ-NAB-ATP and T4PNK - 1.0 U/µL, in buf A after 30 min incubation. Conditions of chromatography: 70 µL Polysil SA column, linear gradient of potassium phosphate 0.0 - 0.3 M in 30% CH$_3$OH, pH 7.5 as eluent; rate 100 µL/min; multiwave detection. (B) Plot of the phosphorylation level of the ON12 versus the γ-NAB-ATP concentration. T4PNK - 1.0 U/µL.

Fig. 4. Photoaffinity labelling of RPA and FEN 1 by 5'-FAB-p-ON17. Reaction mixture in buf. A contained 1.3 µM 3'-[32P] photoreactive primer-template. (Radioactive labelling of photoreactive oligonucleotides was performed via incorporation of [32P]dCMP into 3'-end of the primer by pol β. The reaction mixture in buf. A contained: 10 µM α[32P] dCTP, 1.3 µM primer-template and 0.4 µM pol β. Reaction was continued at 37°C for 30-60 min afterwards reaction mixture was heated at 90°C for 10 min for denaturation of pol β, and then allowed to cool slowly to room temperature; denatured protein was discarded by centrifugation). RPA or FEN-1 were added to the reaction mixtures to final concentration 0.1 - 0.5 µM. Reaction mixtures were placed on ice and then irradiated for 15 min (λ> 280 nm). The products of UV-crosslinking were separated by SDS-PAGE and visualized by autoradiography. The positions of unmodified proteins (according to Coomassie staining) are indicated on the right.
increased enzyme concentration like the case described in (Khlimankov et al., 2001b) for the chemically synthesized 5’-derivative. The easiness, high yield of the goal product and the absence of the side products are the obvious advantages of the developed approach in comparison with chemical synthesis, especially in those cases when wide range of photoreactive oligonucleotides with different sequences is necessary.

Acknowledgements

This work was supported in part by a grant INTAS-96-1778, RFBR № 01-04-48895, 01-03-32439 and NATO Collaborative Linkage Grant-No 978233.

References