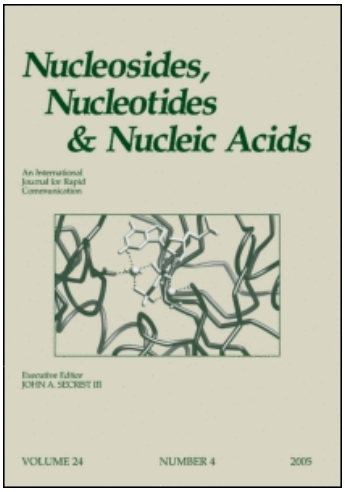

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Magda A. Dineval[a]; Ivan G. Ivanov[b]; Dimiter D. Petkov[a]
[a] Laboratory BioCatalysis, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria [b] Department of Gene Regulations, Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia, Bulgaria

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Pα-METHYL DEOXYNUCLEOSIDE TRIPHOSPHATES AS SUBSTRATES FOR *E. COLI* DNA POLYMERASE I IN A TEMPLATE-DIRECTED SYNTHESIS OF DNA

Magda A. Dineva[1], Ivan G. Ivanov[2] and Dimiter D. Petkov[1]*

[1] Laboratory BioCatalysis, Institute of Organic Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria; [2] Department of Gene Regulations, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

ABSTRACT Pα-methyl deoxynucleoside triphosphates are used as substrates for *E. coli* DNA polymerase I in template-directed polymerase reactions. It is shown that the modified compounds are incorporated together with the unmodified deoxynucleoside triphosphates into DNA under both nick-translation and Klenow reaction conditions.

Antisense oligo- and polynucleotides are widely used as specific compounds for gene inhibition in laboratory experiments and the therapy of viral and cancer diseases[1-4]. Numerous studies have shown that chemically modified synthetic oligodeoxynucleotides have increased specificity, resistance to extra and intracellular enzymes and enhanced penetration through cell membranes. A promising modification is the substitution of one or more of the natural 3', 5'-phosphodiester bonds by their methylphosphonodiester analogues[1,5]. The P-methylphosphonodiester bond however is chiral and can either exist in Sp or Rp configuration. There are experimental results showing that the configuration at the chiral centre might interfere with the biophysical and biochemical properties of the antisense DNA[2,4,6]. These results justify the necessity of a stereospecific method for the synthesis of P-chiral polydeoxynucleotides.

In general, the chemical approach is unreliable for the synthesis of oligonucleotides of high optical purity. The highest optical purity per bond reported so far for chemically synthesized oligonucleotides containing P-methyl

internucleotide bonds is 96%[7]. Even that stereoselectivity is insufficient for the synthesis of a diastereoisomerically pure oligonucleotide. For example, a purity of 96% per bond means that the overall purity of a 10-mer is $0.96^{10} = 0.66$. Thus, the use of homochiral catalysts, such as enzymes, is still the method of preference for the creation of P-chiral nucleotide bonds[8,9].

A successful stereospecific synthesis of oligonucleotides bearing methylphosphonodiester bonds has been accomplished by terminal deoxynucleotidyltransferase using P$^{\alpha}$-methyl thymidine triphosphate (TTP_{Me}) as a substrate[10]. The TTP_{Me} has also been recognized as a substrate analogue of TTP in several DNA polymerase template-directed reactions[11,12].

In a previous study we have reported a method for chemoenzymic synthesis of P$^{\alpha}$-methyl dCyd, dAdo and dGuo triphosphates ($dCTP_{Me}$, $dATP_{Me}$, $dGTP_{Me}$)[13]. This study enabled us to investigate the capability of both forms of *E. coli* DNA polymerase I, holoenzyme and large (Klenow) fragment (KF) to incorporate P$^{\alpha}$-methyl deoxynucleoside triphosphates in DNA in a template-directed reaction.

EXPERIMENTAL

E. coli DNA polymerase I (Klenow fragment) reaction was carried out in 15 µl 10 mM Tris-HCl buffer pH 7.9, containing 5 mM $MgCl_2$, 0.4 mM dithiothreitol, 10 µM *Hind*III/*Hind*III 600 b.p. fragment released from the plasmid pIBMP1R9 (purified by low-melting agarose gel electrophoresis), 100 µM of each of the three dNTP and 200 µM of the fourth modified $dNTP_{Me}$, 1.5 pmol [α-^{32}P] dCTP and 1 unit of KF (Amersham). Samples containing the four natural (unmodified) dNTP's were run in parallel and used as controls. After an incubation for 30 min. at 30°C, aliquots of 2 µl were spotted onto nitrocellulose membranes (Bio-Rad) and counted in a Beckman LC 1801 scintillation counter before and after consecutive washings with 5% trichloroacetic acid/ 20% $Na_4P_2O_7$ and 70% C_2H_5OH.

Nick translation was carried out in 100 µl Tris-HCl buffer pH 7.5 containing 1 mM dithiothreitol, 50 µg serum albumin, 10 mM $MgCl_2$ or 0.5 mM $MnCl_2$, 1 µg plasmid pUC19, 2.5 µM of each of the three dNTP and the fourth modified $dNTP_{Me}$, 0.10 µM [α-^{32}P] dCTP, 5 unit *E. coli* DNA polymerase I (New England BioLabs) and $5x10^{-5}$ µg deoxyribonuclease I (New England BioLabs) at 37°C. Control reaction mixtures containing the four unmodified dNTP's or only three of them (blank controls) were run in parallel. Aliquots of 2 µl withdrawn from the

reaction mixtures at appropriate time intervals were analyzed using the above mentioned procedure.

Natural (unmodified) dNTP's were from the New England BioLabs and the TTP_{Me}, $dCTP_{Me}$, $dATP_{Me}$ and $dGTP_{Me}$ were synthesized and purified as previously described[12,13].

RESULTS AND DISCUSSION

The incorporation of $dATP_{Me}$, $dGTP_{Me}$ or $dCTP_{Me}$ in DNA was studied in a reaction of 3'-recessive ends extension of a *Hind*III/*Hind*III DNA fragment by KF (FIG. 1).

Methylphosphonodiester bond formation was followed by the incorporation of [α-^{32}P] dCTP using reaction mixtures in which each one of the four $dNTP_{Me}$ was substituted for the corresponding natural dNTP. The results presented in TABLE 1 show that the efficiency of single point incorporation was 64%, 67% and 87% for the $dATP_{Me}$, $dGTP_{Me}$ and $dCTP_{Me}$ respectively. The simultaneous utilisation of $dATP_{Me}$ and $dGTP_{Me}$ lead to the incorporation of 50% [α-^{32}P] dCTP. The latter result revealed the possibility for formation of two consecutive methylphosphonodiester bonds through the copolymerisation of two different $dNTP_{Me}$'s. Molecular size of the labelled DNA (*Hind*III/*Hind*III) fragment was analyzed by polyacrylamide gel electrophoresis followed by autoradiography. No difference was found in comparison with the fragments labelled in the presence of natural dNTP's only (data not shown).

It is known that the methyl group at the methylphosphonic internucleotide bond causes partial distortion of the double helix in DNA[14]. Most likely, this distortion yields steric and conformational changes in the ternary complex KF.DNA.dNTP. These changes seem to deteriorate the formation of the next internucleotide bond which lead to a insignificant decrease in the DNA polymerase activity (TABLE 1).

The ability of *E. coli* DNA polymerase I to incorporate methylphosphonate nucleoside triphosphates in DNA was studied also under nick translation reaction conditions using the holoenzyme. The reactions were run in the presence of each one of the $dNTP_{Me}$'s and the other three unmodified dNTP and plasmid DNA as a template. This model made possible to evaluate enzyme activity using the same primer-template DNA system for the $dNTP_{Me}$'s. The equal reaction conditions allowed us to compare the substrate activity of the $dNTP_{Me}$'s used in this study with that of the TTP_{Me} as reported in the literature[11,12]. The efficiency of DNA

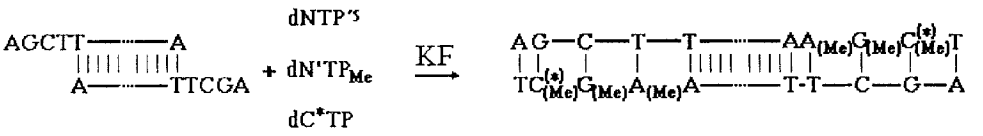

FIG. 1

TABLE 1. Efficiency of formation of methylphosphonodiester bonds utilising $dATP_{Me}$, $dGTP_{Me}$ or $dCTP_{Me}$.

dNTP and $dN'TP_{Me}$ in the reaction mixture	Incorporation of [α-^{32}P] dCTP in DNA (%)*
dCTP, dGTP and TTP	9
dCTP, dGTP, TTP and $dATP_{Me}$	64
dCTP, dATP and TTP	13
dCTP, dATP, TTP and $dGTP_{Me}$	67
dCTP, TTP, $dATP_{Me}$ and $dGTP_{Me}$	50
dATP, dGTP, TTP and $dCTP_{Me}$	87

* Percentage of incorporation is determined on the basis of a control reaction mixture containing the four dNTP and [α-^{32}P] dCTP. All values are the average of triplicate determinations. One percent is equal to 118 cpm [α-^{32}P] dCTP incorporated into DNA. S.D. is less than 10%.

polymerase I to utilized $dNTP_{Me}$'s was assayed by the incorporation of [α-^{32}P]dCTP using two co-factors, Mg^{2+} and Mn^{2+}.

The results presented in TABLE 2 show that the incorporation of the radioactive marker in the presence of TTP_{Me}, $dGTP_{Me}$ and $dATP_{Me}$ was two to three times higher when Mn^{2+} was used instead of Mg^{2+} (TABLE 2, FIG. 2, 3). These results are consistent with the literature data showing that the substrate specificity of DNA polymerase I towards dNTP analogues decreases in the presence of Mn^{2+} [15].

TABLE 2. Incorporation of TTP_{Me}, $dGTP_{Me}$ and $dATP_{Me}$ by DNA polymerase I in the presence of Mn^{2+} and Mg^{2+}.

$dNTP_{Me}$	Mn^{2+}		Mg^{2+}	
	k	A%	k	A%
TTP_{Me}	1.9	50	3.6	15.0
$dATP_{Me}$	4.2	22	4.8	13.8
$dGTP_{Me}$	3.0	29	4.3	14.1

$k=(v_{o\ inc.\ control}/v_{o\ inc.\ dNTPMe})$;

$A\%=(max.\%_{\ inc.\ dNTPMe}/max.\%_{\ inc.\ control})x100$

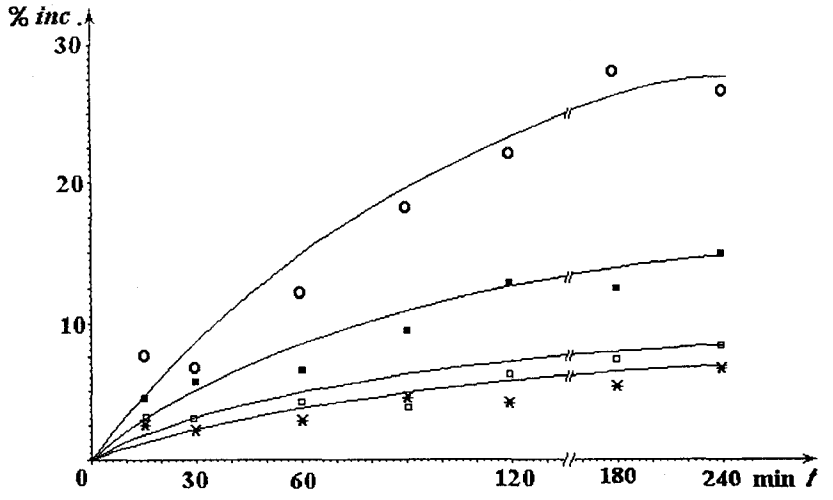

FIG. 2. Kinetics of DNA polymerase I catalyzed incorporation of [α-^{32}P] dCTP in the presence of Mn^{2+} ions. Nick-translation reactions were carried out with either dNTP's (○) or $dNTP_{Me}$'s substituted for one of the natural dNTP's: (■) TTP_{Me}; (□) $dGTP_{Me}$; (✳) $dATP_{Me}$. One percent is equal to 220 cpm incorporated into DNA. The residual radioactivity of the blank controls (run without one of the four dNTP's) was less than 1%. Each point represents an average value of three independent experiments.

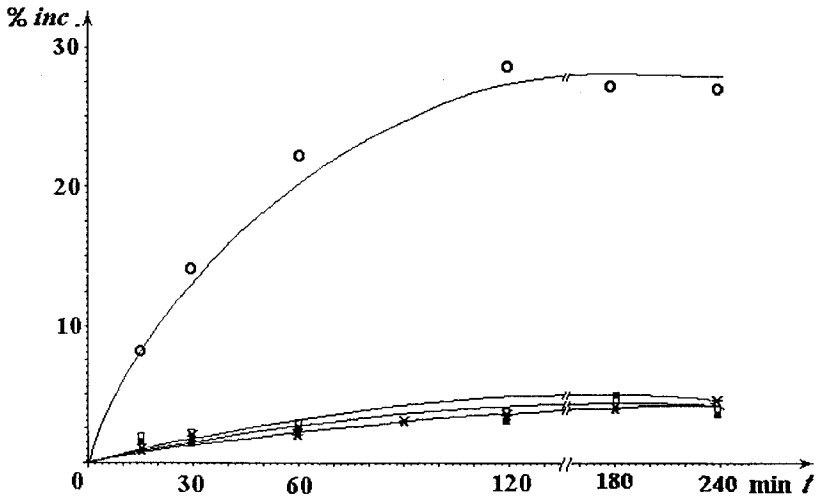

FIG. 3. Kinetics of DNA polymerase I catalyzed incorporation of [α-^{32}P] dCTP in the presence of Mg^{2+} ions. Nick-translation reactions were carried out with either dNTP's (○) or $dNTP_{Me}$'s substituted for one of the natural dNTP's: (■) TTP_{Me}; (□) $dGTP_{Me}$; (⋊) $dATP_{Me}$. One percent is equal to 220 cpm incorporated into DNA. The residual radioactivity of the blank controls (run without one of the four dNTP's) was less than 1%. Each point represents an average value of three independent experiments.

Apparently the lower specificity of the DNA polymerase I in the presence of Mn^{2+} is due to the different coordinating properties of the two Mg^{2+} and Mn^{2+} ions. It is known that Mn^{2+} is a milder complexing agent coordinating not only phosphate oxygens but also with other atoms from the base moiety[16]. It has been proposed that the substitution of Mn^{2+} for Mg^{2+} leads to conformational alterations in the metallo-enzyme.DNA.dNTP complex[17]. Crystallographic data show that metal ions are important entities for the configuration of the enzyme catalytic centre[18]. Apparently the better coordination of the substrate by Mn^{2+} in the matallo-enzyme.DNA.dNTP complex is the reason for the higher DNA polymerase activity in the presence of $dNTP_{Me}$ during the nick-translation reaction.

The results of this study indicate that both the holoenzyme and the large fragment (KF) of *E. coli* DNA Polymerase I recognize the four $dNTP_{Me}$'s as substrates in template-directed DNA polymerase reactions. This observation provides an opportunity for the synthesis of DNA molecules containing chiral methylphosphonodiester bonds for future application.

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