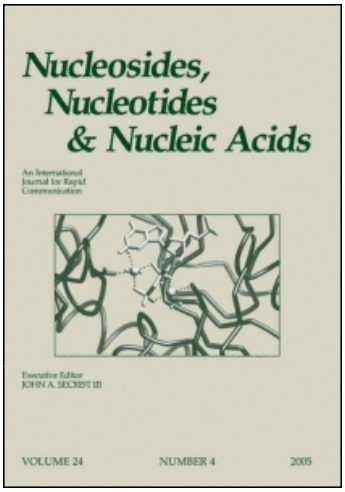

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ASSIGNMENT OF THE ABSOLUTE CONFIGURATION OF P-CHIRAL 5′mRNA CAP ANALOGUES CONTAINING PHOSPHOROTHIOATE MOIETY

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□ *Enzymatic cleavage of the P-chiral diastereoisomers of the 5′ mRNA cap analogue bearing phosphorothioate moiety in alfa position of 5′,5′-triphosphate bridge (m^7 Gppp$_S$G D1 and D2) was performed by human Decapping Scavenger (DcpS) enzyme. Analysis of the degradation products allowed to estimate the absolute configuration at the asymmetric phosphorus atoms in examined compounds via correlation with the R_P and S_P diastereoisomers of guanosine 5′-O-(1-thiodiphosphate) (GDPαS).*

Keywords 5′ mRNA Cap; phosphorothioate; P-chirality; absolute configuration; enzymatic hydrolysis

INTRODUCTION

P-chiral nucleotide analogues have found versatile applications as tools for studying stereochemical aspects of interactions of nucleotides with other biomolecules.[1–3] Phosphorothioate nucleotide analogues, in which one of the non-bridging oxygens is replaced by sulfur, belong to the most intensively investigated and explored group used for this purpose.[3,4] Moreover, introduction of the phosphorothioate moiety into nucleotides improves their resistance against enzymatic cleavage.[4]

Cap structure present at the 5′ end of all eukaryotic mRNAs, consisting of 7-methylguanosine linked to the first nucleotide of mRNA chain via 5′,5′-triphosphate bridge, is a very unique example of biologically significant nucleotide. Synthetic cap analogues serve as invaluable tools for studying

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initiation of protein translation and many other processes concerning mRNA metabolism.[5–9]

We have recently reported the synthesis and some preliminary biological studies of several dinucleotide 5′ mRNA cap (abbr. m^7GpppG) analogues bearing phosphorothioate moiety in either α, β, or γ position of the polyphosphate chain.[10] Due to the presence of chirality center at phosphorus in the phosphorothioate moiety all synthesized compounds exist in a form of two epimers, marked D1 and D2 according to the elution order from HPLC RP column. Among other results it has been shown that the D1 and D2 epimers significantly differ in association constants with the eIF4E protein, which is responsible for specific recognition of the cap structure during translation initiation process.[10] In all cases fluorimetric titration measurements gave substantially higher association constants of eIF4E for isomers D1 than for isomers D2 and unmodified cap analogue. These data could be useful for further evaluation of molecular aspects of interaction between 5′ mRNA cap and eIF4E factor, providing that absolute configuration at phosphorus is known.

In this work we describe assignment of the absolute configuration of the asymmetric phosphorus centre in the cap analogue containing phosphorothioate moiety in the α position of 5′,5′-triphosphate chain (m^7Gppp_SG) (Figure 1).

The idea of the experiment was to determine a correlation between the structure of examined cap analogue and a compound of known configuration that was reported previously, namely guanosine 5′-O-(1-thiodiphosphate) (GDPαS).[11,12] This was achieved by enzymatic hydrolysis of m^7Gppp_SG with human mRNA Decapping Scavenger (DcpS) enzyme, which is a pyrophosphatase specific for the 5′ mRNA cap.[13]

ENZYMATIC HYDROLYSIS WITH DCPS

mRNA Deccaping Scavenger, DcpS, is a member of the histidine triad (HIT) family of hydrolases and is involved in the mRNA 3′ → 5′ degradation pathway.[14] DcpS is responsible for cleavage of short capped mRNAs and free m^7GpppN dinucleotides released after mRNA degradation by exosome, and is unable to cleave the cap structure attached to a long RNA chain.[15]

DcpS catalyses regiospecific hydrolysis of pyrophosphate bond in beta/gamma position of 5′, 5′-triphosphate bridge in mRNA cap.[6] According to that, standard cap analogue (m^7GpppG) is hydrolysed by DcpS in beta/gamma position resulting in m^7GMP and GDP. Assuming that enzymatic digestion of m^7Gppp_sG with DcpS proceeds in the analogical manner, the reaction should result in m^7GMP and one of the isomers of GDPαS (R_P or S_P) dependent on the configuration of the cap analogue. Since the

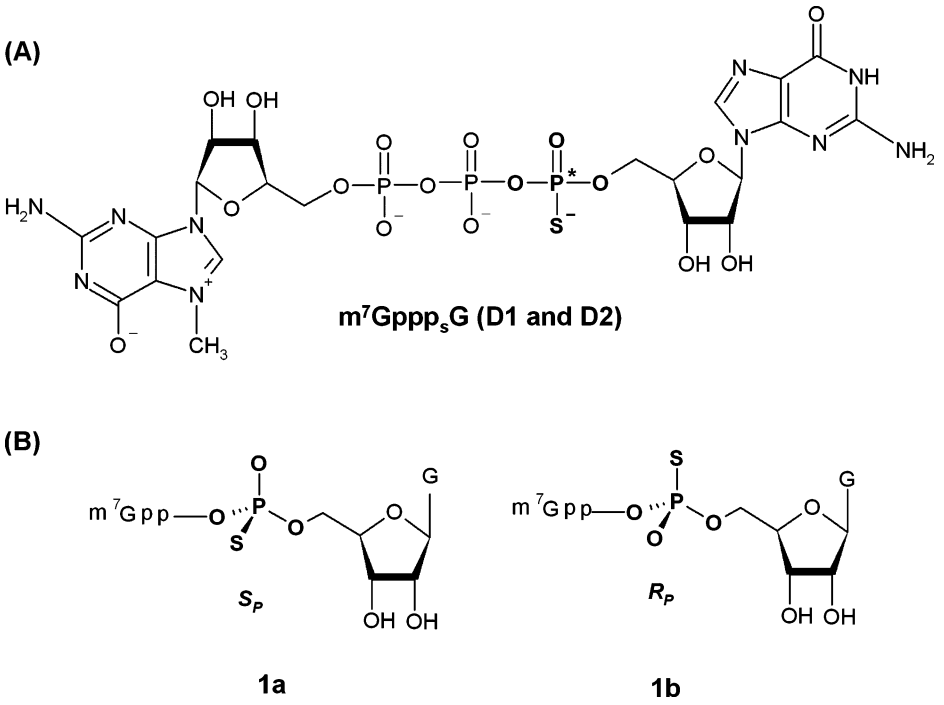

FIGURE 1 Modified 5′ mRNA cap analogue with phosphorothioate moiety in alfa position of the 5′, 5′- triphosphate chain: (A) chemical structure; (B) stereochemical arrangement of substituents around asymmetric phosphorus in S_P and R_P diasteroisomers.

absolute configurations of GDPαS isomers are known,[11,12] the configurations of m^7Gppp_sG isomers would be determined.

Therefore, an enzymatic hydrolysis of m^7Gppp_sG D1 and D2 isomers with human DcpS enzyme was performed. The reaction with DcpS was performed in conditions described below. 20 μl of 1 mM cap analogue solution was added to the 500 μl of buffer containing 20 mM $MgCl_2$ and 60 mM $(NH_4)_2SO_4$. The mixture was shortly incubated at 30°C. Finally, 5–10 μl of hDcpS enzyme was added and further incubated at 30°C. As a control, degradation of standard cap analogue (m^7GpppG) also was performed. Both diastereoisomers, D1 and D2 were hydrolyzed at the similar rate as m^7GpppG. The amounts of the enzyme added were fitted to achieve complete degradation of the standard cap analogue within 40–60 minutes. After 10, 20, 30, and 40 minutes from the start, 100 μl samples were collected, deactivated in 100°C for 3 minutes, and analyzed on analytical reverse-phase HPLC (Supelcosil LC-18-T 4.6 × 250 mm column, buffer A: 0.1M KH_2PO_4 pH = 6.0, buffer B: buffer A/MeOH, 1:1 (v/v), linear gradient of buffer B from 0 to 50% in 30 minutes, UV detection at 254 nm).

RESULTS AND DISCUSSION

As expected, the enzymatic cleavage of both $m^7GpppSG$ isomers with DcpS resulted in two products: m^7GMP and one of two diastereoisomers of GDPαS. Hydrolysis of $m^7GpppSG$ D1 isomer (Rt = 10.3 minutes) (Figure 2B) led solely to m^7GMP (Rt = 6.5 minutes) and GDPαS diastereoisomer with retention time (Rt) = 3.6 minutes. The products of $m^7GpppSG$ D2 isomer hydrolysis were m^7 GMP and GDPαS diastereoisomer with retention time Rt = 5.0 minutes (Figure 2B). RP HPLC profile of the degradation of the standard cap analogue, m^7GpppG, also is shown for comparison (Figure 2B).

It was presumed that degradation of $m^7GpppSG$ D1 gave GDPαS isomer D1 (shorter retention time), the configuration of which had been assigned previously as S_P, whereas the digestion of $m^7GpppSG$ D2 resulted in GDPαS isomer D2 (R_P).[11,12] The outcome was confirmed by several HPLC co-injections of reaction mixtures with standard samples of guanosine 5′-O-monothiophosphate and with chemically synthesized and chromatographically separated samples of GDPαS D1 (identical with product

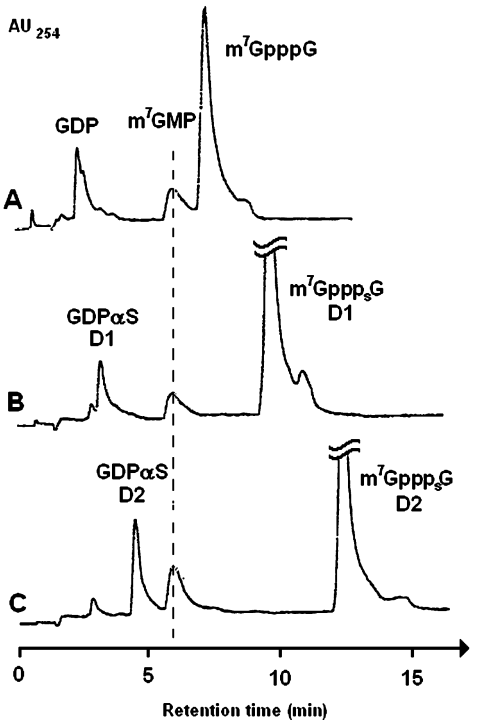

FIGURE 2 Enzymatic hydrolysis by mRNA Deccaping Scavenger enzyme (DcpS). RP HPLC profiles of the reaction mixtures after 10 minutes incubation with enzyme: (A) m^7GpppG, (B) $m^7GpppsG$ D1, and (C) m^7Gppp_sG D2. For the conditions of the reaction and HPLC analysis see the text.

Rt = 3.6 minutes) and GDPαS D2 (identical with product Rt = 5.0 minutes). Formation of GMPS during hydrolytic reaction was not observed. Since the hydrolytic reaction proceeds with participation of β and γ phosphate groups exclusively, there is no possibility of inversion of the configuration at α phosphorus within the process. Hence, configurations around asymmetric phosphorus atoms in $m^7GpppsG$ isomers D1 and D2 have been assigned as S_P (Figure 1, structure 1a) and R_P (Figure 1, structure 1b) respectively.

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