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The effect of divalent cations on the catalytic activity of the human plasma 3′-exonuclease

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Abstract The 3′-exonuclease from human plasma is a soluble form of nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) (EC 3.1.4.1/EC 3.6.1.9). Here, the possibility of divalent cation influence for the 3′-exonuclease activity was investigated using the phosphorothioate congener of oligonucleotide containing all phosphorothioate internucleotide linkages of the [R_P]-configuration ([R_P-PS]-d[T_{12}]) as the substrate for this enzyme. It was found that the 3′-exonuclease is a metalloenzyme, i.e. its phosphodiesterase activity was completely abolished at 0.8 mM concentration EDTA and, in turn, it was restored in the presence of Mg^{2+} or Mn^{2+} ions. In addition, Mg^{2+} can be replaced effectively by Ca^{2+}, Mn^{2+}, or Co^{2+}, but not by Ni^{2+} and Cd^{2+} during the hydrolysis of the phosphorothioate substrate in human plasma. In addition, the mechanism is postulated, by which a single internucleotide phosphorothioate bond of the S_P-configuration at the 3′-end of unmodified phosphodiesters (PO-oligos), or their phosporothioate analogs (PS-oligos) protects these compounds against degradation in blood.

Keywords The human plasma 3′-exonuclease · NPP1 · Catalysis · Phosphorothioates

Introduction

Phosphate-modified DNA analogs, in which one of the non-bridging oxygen atoms of internucleotide phosphate moiety is replaced with a sulfur atom giving rise to phosphorothioates oligonucleotides (PS-oligos), have been widely utilized both in vitro and in vivo studies (Hoke et al. 1991; Stewart et al. 2003; Luganini et al. 2008; Abaza et al. 2008). Given the PS modification represents the most conservative elemental replacement that can be made to the phosphate (Frey and Sammons 1985), and moreover, incorporation of sulfur makes the phosphorus center chiral thereby, the PS internucleotide linkage exists in the [R_P]- or [S_P]-configuration (Fig. 1), a large number studies with stereodefined PS-oligos have been performed to determine the stereochemical course of reactions catalyzed by certain kinases (Orr et al. 1978), nucleotidyl transferases (Eckstein et al. 1977; Sheu and Frey 1978), polymerases (Eckstein et al. 1976; Bartlett and Eckstein 1982),

The experimental work was carried out at the Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies of Polish Academy of Sciences in Lódź.

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endonucleases (Connolly et al. 1984; Grasby and Connolly 1992), and exonucleases (Eckstein et al. 1979; Gupta and Benkovic 1984; Koziolkiewicz et al. 2002). Among enzymes acting in exonucleolytic fashion, much attention has been paid to the 3′-exonuclease present in human plasma (HP) in regard to its phosphodiesterase activity toward unmodified and phosphorothioate oligonucleotides degraded successively from the 3′-end to the 5′-end with releasing of nucleoside-5′-phosphate and 5′-phosphorothioates, respectively (Eder et al. 1991; Koziolkiewicz et al. 1997; Gilar et al. 1998). It is now accepted that the human plasma 3′-exonuclease possesses the [R_P]-selective nature and its digestion mechanism proceeds with retention of configuration at a phosphorus atom, that is, the reaction occurs via a two-step mechanism with participation of a covalent enzyme-substrate intermediate (Gijsbers et al. 2001; Koziolkiewicz et al. 2002). This fact, combined with the result obtained from immunoprecipitation assay with mouse monoclonal antibodies 3E8 binding the native form of NPP1 (also known as plasma cell membrane glycoprotein PC-1, EC 3.1.4.1/3.6.1.9) confirmed that the human plasma 3′-exonuclease is a soluble form of PC-1 belonging to the nucleotide pyrophosphatases/phosphodiesterases (NPPs) family (Wojcik et al. 2007), widely distributed and phylogenetically conserved membrane-associated or secreted proteins involved in regulation of various physiological processes, including bone mineralization, calcification of ligaments and joint capsules, signaling by nucleotides and insulin, and the differentiation and motility of cells (Bollen et al. 2000; Goding et al. 2003; Stefan et al. 2005). Multiple roles of NPPs are consequence, at least in part, of their action on distinct substrates like nucleotides and nucleotide derivatives as well as lysophospholipids and choline phosphate esters.

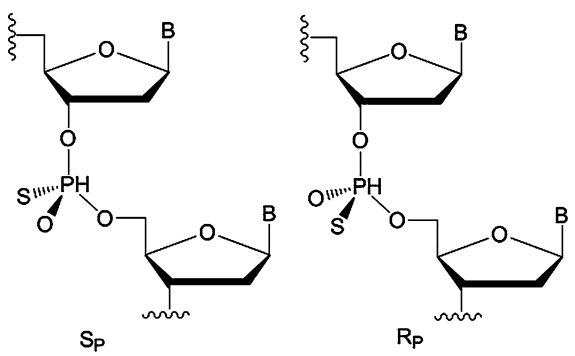

Fig. 1 The P-chiral phosphorothioate internucleotide bond of the S_P- and R_P-absolute configuration, respectively

Since cytotoxic and/or proliferative effect of deoxyribonucleoside-5′-monophosphorothioates (dNMPSs) resulting from exonucleolytic degradation of PS-oligos has been reported (Vaerman et al. 1997; Koziolkiewicz et al. 2001), an increasing interest in the human plasma 3′-exonuclease, able to hydrolyze of extracellular nucleotides, has been observed. Whereas the stereochemical mechanism and the substrate specificity of the 3′-exonuclease have been well characterized (Eder et al. 1991; Koziolkiewicz et al. 1997, 2002; Gilar et al. 1998), nothing is known about its three-dimensional structure. This prompted us to carry out functional studies aiming at the determination of divalent cations which contribute to catalysis of PO- and PS-oligos in HP.

Materials and methods

Materials

T4 polynucleotide kinase (EC.2.7.1.78) was supplied by Amersham (Little Chalfond, U.K.). [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc. (Irvine, CA, USA). Snake venom phosphodiesterase (sv PDE, EC.3.1.15.1) was obtained from Boehringer Mannheim (Germany). Nuclease P1 (nP1, EC.3.1.30.1) from *Penicillium citrinum,* $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $CdCl_2$, and $NiCl_2$ (>99.99%) were purchased from Sigma-Aldrich.

HP was isolated from blood containing 0.38% sodium citrate by centrifugation at 3000 rpm for 5 min. Plasma aliquots were carefully withdrawn, leaving the untouched packed cell.

The PO-oligo **1** (Table 1) was prepared by the phosphoramidite method on an ABI 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The PS-oligos **2** and **3** were synthesized using the oxathiaphospholane method, as described elsewhere (Stec et al. 1995). The PS-oligo **2** contained exclusively R_P-internucleotide bonds, whereas the PS-oligo **3** contained ten consecutive R_P bonds followed by a single S_P linkage located at the 3′- end. Purification of the oligonucleotides was carried out by two-step reverse phase (RP) HPLC (DMT-on and DMT-off) (Zon and Stec 1991), and their purity was confirmed by 20%/7 M urea polyacrylamide gel electrophoresis

Table 1 Oligonucleotides used in the 3′-exonuclease studies

Sequence 5′ → 3′	$t_{1/2}$ (min)[a]	$t_{1/2}^{Mg}$ (min)[b]	$t_{1/2}^{Mn}$ (min)[c]
[PO]-d[T_{12}] (**1**)	20	5	40
[R_P-PS]-d[T_{12}] (**2**)	240	120	120
PS-[All-R_P]-d[T_{10}]T_{Sp}T (**3**)	ND	ND	ND

The 5′-labeled oligonucleotides (5 μM) were incubated in 50% HP [a]without metal ions and [b]in the presence of $MgCl_2$ or [c]$MnCl_2$ (10 mM), at 37°C. At various times 10 μl aliquots containing a mixture of oligonucleotides were withdrawn, and analyzed by denaturing PAGE followed by autoradiography. The $t_{1/2}$ values were calculated on the basis of the densitometric data. All experiments were performed in triplicate with the standard deviation <15% among triplicates

ND cleavage was not detected after 8 h of incubation

(PAGE). The diastereomeric purity of the PS-oligos was confirmed by enzymatic degradation using R_P- and S_P-specific enzymes: sv PDE and nP1, respectively (Stec et al. 1995).

Enzyme assays

The oligonucleotides used in these studies were 5′-end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. A mixture (20 μl) containing 10 mM Tris–Cl (pH 8.5), 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 1.0 nmol oligonucleotide, 1 μl (10 μCi) [γ-^{32}P] ATP, and T4 polynucleotide kinase (5 units) was incubated for 1 (in the case of the PO-oligo **1**), and 3 h (in the case of the PS-oligos **2** and **3**) at 37°C, then heat denatured and stored at −20°C.

The mixtures containing HP (50%), phosphate-buffered saline (PBS), and $MgCl_2$ or $MnCl_2$ (10 mM) were incubated with the ^{32}P-radiolabeled oligonucleotides **1–3** (5 μM) at 37°C. At various times, 10 μl aliquots were withdrawn and heated at 95°C for 2 min. Then, 100 μl water was added to each sample. After vigorous shaking, the protein precipitates were spun down and the aqueous solutions were lyophilized in a Speed Vac rotary evaporator (Savant Instruments, Farmingdale, NY). The resultant samples were dissolved in formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol (10 μl), and analyzed by 20%/7 M urea PAGE. The half-life ($t_{1/2}$) values depicted in Table 1 were calculated on the basis of densitometric analysis of the autoradiograms.

Measurements of the 3′-exonucleolytic activity of HP toward ^{32}P-radiolabeled PS-oligo **2** (5 μM) in the presence of $MgCl_2$, $MnCl_2$ (0–40 mM), $CaCl_2$, $CoCl_2$, $CdCl_2$, and $NiCl_2$ (10 mM) were carried out at 37°C for 4 h, and analyzed as described above.

The effectiveness of Mg^{2+} and Mn^{2+} ions in restoring activity of the human plasma 3′-exonuclease, previously preincubated with 0.8 mM ethylenediaminetetraacetic acid (EDTA) for 10 min at 37°C (the enzyme activity was completely inhibited), was investigated in mixtures containing the ^{32}P-radiolabeled PS-oligo **2** (5 μM), 50% HP, PBS, and the increasing concentrations either of $MgCl_2$ or $MnCl_2$ (0–40 mM). The aliquots were incubated at 37°C for 4 h. Products of the oligonucleotide degradation were recovered from the incubation mixtures and analyzed as described above. For control neither EDTA nor divalent cations were added to the reaction mixture containing the 5′-labeled oligomer **2** (5 μM), 50% HP, and PBS.

Results and discussion

Restoring activity of EDTA-treated HP in the presence of Mg^{2+} or Mn^{2+} ions

The human plasma 3′-exonuclease has been identified as a soluble form of NPP1 that releases nucleoside-5′-phosphates or 5′-phosphorothioates from the 3′-end of PO- and PS-oligos in blood, respectively (Wojcik et al. 2007). Several lines of evidence indicate that divalent cations are required for the catalytic activity of NPP1, NPP2 (discovered as autotaxin or PD-1-α), and NPP6 (Rebbe et al. 1991; Oda et al. 1993; Gijsbers et al. 2001; Duan et al. 2003; Sakagami et al. 2005). Interestingly, the catalytic activity of NPP6 is inhibited by EDTA, but it is not restored by the divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+} (Sakagami et al. 2005). On the other hand, it has been reported that NPP7 (the human intestinal alkaline sphingomyelinase/alk-SMase), unlike the above mentioned members of the NPP family, is not stimulated by Mg^{2+} and Ca^{2+} ions, and moreover, its activity is abolished by Zn^{2+} (Duan et al. 2003). Given different NPP proteins have different requirements for divalent cations, the determination of these essential for the catalytic activity of the human plasma 3′-exonuclease was a

subject of our particular interest. Since our earlier studies revealed that the 3′-exonuclease present in HP is R_P-selective, we employed the phosphorothioate oligomer [R_P-PS]-d[T_{12}] (**2**) containing exclusively [R_P]-internucleotide bonds (Table 1), as the substrate to compare the effects of Mg^{2+} and Mn^{2+} ions on the phosphodiesterase activity of the enzyme. For this purpose the 5′-labeled dodecanucleotide **2** was incubated in 50% HP with increasing concentrations $MgCl_2$ or $MnCl_2$ (from 0 to 40 mM) at 37°C for 4 h (that is $t_{1/2}$ for the oligomer **2** incubated alone in 50% HP). The reaction progress was analyzed by denaturing PAGE, and vizualized by autoradiography. The quantification of the obtained data showed that the cleavage of the oligomer **2** in the presence of $MgCl_2$ and $MnCl_2$ at a 10 mM concentration was enhanced approx. 20 and 30%, respectively, compared to the control (Fig. 2). Further increase of $MgCl_2$ concentration (>10 mM) had no effect on the enhancement of the 3′exonuclease-catalyzed cleavage reaction, whereas it was declined approx. 10% at higher concentration of $MnCl_2$ (40 mM). It should be noted that contrary to some other enzymes, the 3′-exonuclease does not change of the sequence-specific cleavage activity in the presence of Mn^{2+} ions at millimolar concentrations, and a typical ladder of products characteristic for exonucleases was observed as the degradation products.

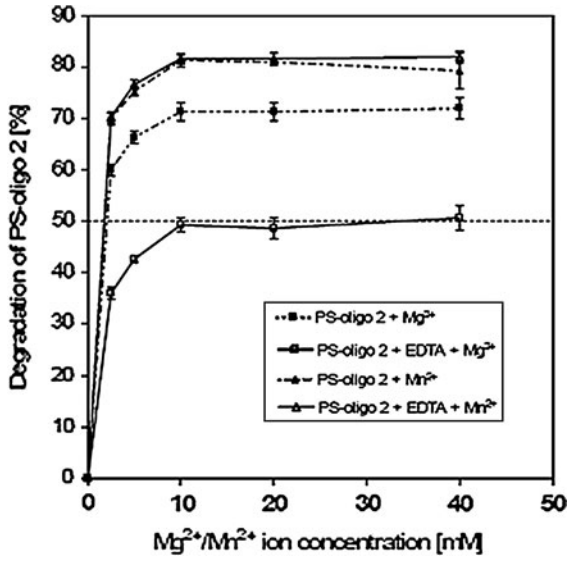

Fig. 2 The effect of $MgCl_2$ or $MnCl_2$ concentration on catalysis of the PS-oligo **2** cleavage by the 3′-exonuclease

To confirm that Mg^{2+} and Mn^{2+} ions are essential for the catalytic activity of the 3′-exonuclease, we treated HP with 0.8 mM EDTA resulting in a complete loss of the 3′-exonucleolytic activity in the reaction with the 5′-labeled phosphorothioate substrate **2**, and subsequently, either of Mg^{2+} or Mn^{2+} ions, in the range of 0–40 mM, were added to the reaction mixture. We found that upon incubation of the metal ion-free enzyme with Mn^{2+} ions, used at concentrations up to 40 mM, the cleavage extent of the substrate was completely restored to the values obtained with the enzyme incubated with the same concentrations of Mn^{2+}, but without EDTA pretreatment (Fig. 2). However, when increasing amounts of Mg^{2+} ions were added to the solution containing EDTA-treated the 3′-exonuclease, only the Mg^{2+}-mediated restoration of the phosphodiesterase activity to the control level (without the addition of any divalent cations) was observed (Fig. 2). This result is not surprising, since a partial recovery of the activity of EDTA-inhibited NPP1 in the presence of Mg^{2+} ions has been found, although at lower cation concentrations (Gijsbers et al. 2001), and, since it is known that the 3′-exonuclease is a soluble form of NPP1 (Wojcik et al. 2007). Taken together, the loss of the phosphodiesterase activity upon treatment with the metal ion chelator that can be restored by the addition of divalent cations such as Mg^{2+} or Mn^{2+} indicates that the human plasma 3′-exonuclease is a metalloenzyme containing Mg^{2+} or/and Mn^{2+} ions in the catalytic site which participate in hydrolysis of [R_P]-PS-oligos. In addition, our results indicate that the hydrolysis of the phosphorothioate substrate in HP is the most efficient in the presence of 10 mM Mg^{2+} or 10 mM Mn^{2+} ions, thus, the enzyme responsible for PS-oligos degradation in blood is Mg^{2+}/Mn^{2+}- dependent.

To determine which of divalent cations tested, Mg^{2+} whether Mn^{2+}, affects stronger the phoshodiesterase activity of the 3′-exonuclease, we carried out additional experiments with the use of the oligonucleotide substrates **1** (PO-oligo) and **2** (PS-oligo). The compounds were incubated in HP in the presence of both $MgCl_2$ and $MnCl_2$ (used at 10 mM each), for 1 and 2 h (for **1**), or for 4 and 8 h (for **2**), at 37°C. The different incubation times reflect different susceptibility of the oligonucleotides **1** and **2** to the enzymatic degradation (Wojcik et al. 2007). The products of the oligonucleotide degradation were recovered from the

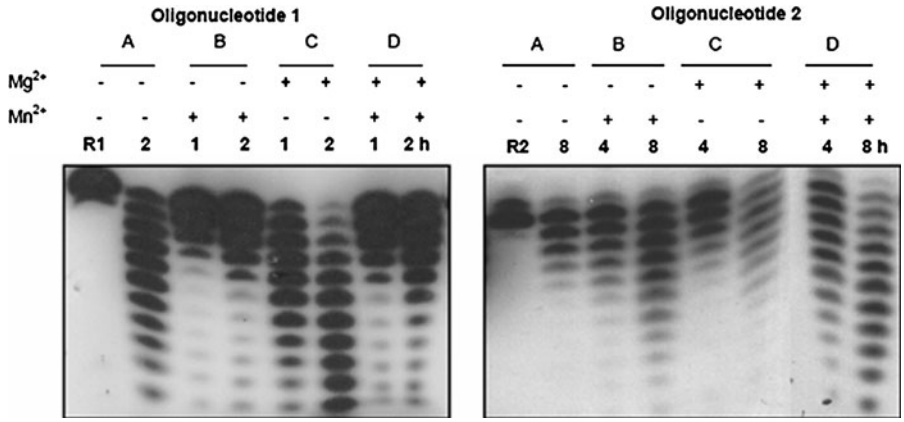

Fig. 3 Effect of Mg^{2+} and Mn^{2+} ions on degradation of the oligonucleotides **1** and **2** in HP. The ^{32}P-labeled oligonucleotides **1** and **2** were incubated in 50% HP alone (*A*) and in the presence of Mn^{2+} (*B*) Mg^{2+} (*C*) the mixture of Mn^{2+} and Mg^{2+} (*D*) Aliquots were withdrawn at given times, and analyzed by denaturing PAGE. *R1* and *R2* refer to the reference 5′-labeled samples (no HP added) dissolved in PBS and incubated at 37°C for 2 and 8 h, respectively

incubation mixtures and analyzed as described above (Fig. 3). It was found that the cleavage of the substrates **1** and **2** in the presence of the mixture of $MgCl_2$ and $MnCl_2$ was similar to that obtained in HP, supplemented only Mn^{2+} ions at 10 mM concentration, suggesting that this effect might be caused by a competition of Mn^{2+} with Mg^{2+} ions for binding to the same metal-binding site on the enzyme (Fig. 3).

The catalytic activity of the 3′-exonuclease in the presence of other divalent cations

To further investigate the possibility of cation dependence for the 3′-exonuclease, we determined of the cleavage of the phosphorothioate substrate **2** in the presence of several other divalent cations belonging to the alkaline earth ions (Ca^{2+}) and the transition metal ions (Co^{2+}, Cd^{2+}, Ni^{2+}). For this purpose the 5′-labeled substrate **2** (5 μM) was incubated in HP with each tested ion metal (used at 10 mM concentration) at 37°C for 4 h (this is $t_{1/2}$ for the oligomer **2** incubated in HP alone) and products of the oligonucleotide degradation were analyzed by PAGE in 20% denaturing gels. The quantification of autoradiograms showed that in the case of the reaction performed in the presence of Ca^{2+} as the sole divalent cation, the extent of the degradation of the PS-oligo **2** was identical to that in the presence of Mg^{2+} (Fig. 4). It may be surprising in the context of the larger ionic radius (0.99 Å), an expanded coordination number of seven, and a lower charge density of calcium ions compared to magnesium which the ionic radius and the coordination number are 0.86 Å and six, respectively (Cowan 1998). Since structural data are not available for the 3′-exonuclease, it is unknown where Ca^{2+} ions bind and how they act to increase the phosphodiester activity of the enzyme. The possible explanation would be that binding of Ca^{2+} ions to a metal-binding region(s) of the 3′-exonuclease enhances a local folding of the molecule resulting in the stabilization of its conformation, thereby affecting the enzymatic activity of the enzyme. This hypothesis was supported by our previous studies (unpublished data), in which the influence of Ca^{2+} and Mg^{2+} ions on the protection of the enzyme activity against thermal denaturation was investigated. This was found that the presence of Ca^{2+} ions, but not Mg^{2+} ions, improved the 3′-exonuclease stability, implying that Ca^{2+} ions can play a predominant role in the conformational stabilization of the 3′-exonuclease, whereas Mg^{2+} ions appear to be essential for the catalytic activity of the enzyme. This observation is in agreement with the finding of Belli et al. (1994), who showed the conformational stabilization of NPP1 by Ca^{2+} ions, which probably bind to a functional variant of EF-hand motif in NPP1. When 10 mM $CaCl_2$ was replaced with 10 mM $CoCl_2$, no changes in the extent of the degradation of the oligomer **2** were

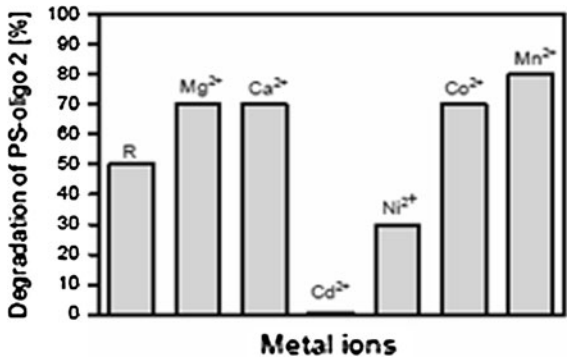

Fig. 4 Effect of metal ions on hydrolytic activity of the plasma 3′-exonuclease. The 5′-labeled substrate (5 μM) was incubated in 50% HP, supplemented Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, or Cd^{2+} at 37°C for 4 h. The resulting products of degradation were analyzed by denaturing PAGE followed by autoradiography and quantitative phosphorimaging. *R* refers to the reference 5′-labeled sample incubated in 50% HP in the absence of metal ions at 37°C for 4 h. All values are the average of two measurements and have maximum error of ±10%

observed (Fig. 4). However, it should be noted that the effect of Co^{2+} ions on catalysis was concentration-dependent, and the hydrolytic process was slowed down when the metal ion concentration was ≥20 mM (data not shown).

Among the tested metal cations from the transition metal group, both Ni^{2+} and Cd^{2+} ions were inhibitors, but Ni^{2+} had little effect on repressing of the enzyme activity (approx. 20%), whereas Cd^{2+} ions resulted in complete inhibition of catalysis, thus, no detectable substrate cleavage over 4 h was observed (Fig. 4). The results obtained for four different thiophilic metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+}) can be explained in a rational way if one assumes that their decreased inhibitory effect, in the following order: $Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$, is correlated with the decreased stability constants for metal-sulfur complexes: $Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$ (Irwing and Williams 1953; Bond et al. 1986). An alternative explanation includes steric effects for the Cd^{2+} ion resulting from its larger ionic radius (1.03 Å) as compared to those of Ni^{2+} (0.83 Å), Mn^{2+} (0.80 Å), or Co^{2+} (0.79 Å) that could cause the perturbation of the catalytic site of the enzyme, previous already perturbed by the bulky sulfur substitution (the radius of sulfur atom is approx. 0.45 Å larger compared to that of the oxygen atom).

Stability of PO- and PS-oligos in HP in the presence of Mg^{2+} or Mn^{2+} ions

In order to better characterize the role of Mg^{2+} and Mn^{2+} ions in degrading PO- and PS-oligos in HP, we measured the cleavage of the 5′-labeled substrates **1** and **2** in HP with either Mg^{2+} or Mn^{2+} as the only divalent cation used at 10 mM concentration. As shown in Table 1, the $t_{1/2}$ value for the PO-oligo **1** incubated with $MgCl_2$ was reduced by fourfold compared to that obtained for the same substrate, but incubated without Mg^{2+} ions. By contrast, under the same conditions, Mn^{2+} ions inhibited the 3′-exonuclease-catalyzed cleavage reactions of the oligomer **1** by twofold, suggesting that there is a correlation between the cleavage efficiency of the PO-oligos in HP and different affinity of Mg^{2+} and Mn^{2+} ions for oxygen, according to the Hard and Soft, Acid and Base (HSAB) rule (Pearson 1968). Since the site-specific sulfur substitution disrupts specific magnesium interactions to the non-bridging phosphate oxygens (the thio-effect) and, since it can be rescued with metal ions softer than Mg^{2+} like Mn^{2+}, Zn^{2+}, or Cd^{2+} (the rescue effect), P-chiral phosphorothioates have been widely used in numerous mechanistic studies (Warnecke et al. 1996; Zhou et al. 1998; Wang et al. 1999). In this work we have shown that the cleavage efficiency of the PS-oligo **2** in HP containig Mg^{2+} ions was 24-fold lower compared to that of the all-oxygen control **1** incubated in HP in the presence of Mg^{2+} (Table 1). Thus, the replacement of the *pro*-R_P oxygen with sulfur at the unmodified all-phosphate-containing oligomer causes the reduction in the catalytic activity of the 3′-exonuclease toward the all-R_P phosphorothioate **2** in the presence of Mg^{2+} (the thio effect). When Mn^{2+} was used in the place of Mg^{2+}, the $t_{1/2}$ value for the PS-oligo **2** was approx. threefold lower than that for the unmodified reference **1** incubated with Mn^{2+} ions (Table 1). The slight reduction of the catalytic activity of the 3′-exonuclease toward the all-R_P phosphorothioate **2** in the presence of Mg^{2+} ions, and moreover, the lack of Mn^{2+} rescue of this thio effect argue against a direct coordination of a metal ion with the *pro*-R_P oxygen of the phosphodiester bond at the cleavage site of a single stranded DNA.

For comparison, we further conducted analogous experiments with the PS-oligo **3** possessing ten consecutive R_P bonds and a single S_P bond at the

3′-end of PS-oligo. It has been earlier demonstrated that the single [S_P]-phosphorothioate linkage located at the 3′-end both the PS-oligo **3** and the chimeric oligomer [PO/S_P-PS]-d[$T_{PO}C_{PO}T_{PO}T_{PO}T_{PO}T_{PO}T_{PS}C$] (where PO and PS refer to the internucleotide phosphate and phosphorothioate bonds, respectively) can effectively protect these compounds against the hydrolysis in HP (Wojcik et al. 2007). In this study, in contrary to the PS-oligo **2**, no degradation of the oligonucleotide **3** was detected in HP supplemented with Mg^{2+} ions within 8 h of incubation (Fig. 5). This "classical" thio effect observed for the oligomer **3** suggests that the *pro*-S_P oxygen plays a much more critical role than does the *pro*-R_P oxygen atom in the 3′-exonuclease-mediated cleavage of a single stranded DNA. However, the replacement of 10 mM Mg^{2+} ions by 10 mM Mn^{2+} ions did not rescue of the cleavage of the single [S_P]-phosphorothioate linkage at the 3′-end of the oligonucleotide **3**, implying the lack of coordination of the metal cation to the *pro*-S_P oxygen atom. On the other hand, we cannot rule out the possibility that inability of thiophilic metal ion to rescue catalysis results from steric constrains imposed by the bulkier sulfur atom which can alter the geometry of metal ion interactions (Brautigam and Steitz 1998).

Taking into account that the double-displacement reaction mechanism for NPPs proposed by Gijsbers et al. (2001) includes the attack of the metal ion 2 (Me2)-activated hydroxyl group of the catalytic-site T238 on the phosphate group of the incoming substrate resulting in the formation of a covalent enzyme-nucleoside monophosphate (NMP) intermediate (the first catalytic step) and, in turn, the attack of the metal ion 1 (Me1)-activated water molecule on the formed enzyme-NMP intermediate leading to the regeneration of the catalytic-site T238 and the generation of a phosphorylated product (the second catalytic step), it may be hypothesized that the *pro*-S_P oxygen of the phosphate group located at the 3′-end of the oligonucleotide could interact with the Me2 (the metal ion involved in binding of D200, D405, H406 and the catalytic-site T238) enabling the formation of the enzyme-substrate complex, in which T238 holds a position opposite to the leaving group of the NMP-ester substrate. We can assume that replacement of the *pro*-S_P oxygen with the bulky sulfur atom might disrupt its coordination to Me2 causing changes in the catalytic site architecture, which in consequence could prevent a nucleophilic attack of the hydroxyl group of T238 on the α-phosphate of the substrate (the inhibition of the first step of the reaction) (Fig. 6). Thus, the above-mentioned hypothesis could explain the resistance of S_P diastereomer located at the 3′-end of the oligonucleotide to the hydrolysis in HP.

In conclusion, we demonstrate that the 3′-exonuclease is not unique in its divalent metal requirement among the NPPs and its phosphodiesterase activity toward PS-oligo was enhanced in the presence of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} ions, providing a baseline for understanding of the inherent metal ion preferences for the enzyme in the process of degradation of PS oligonucleotides in HP and suggesting

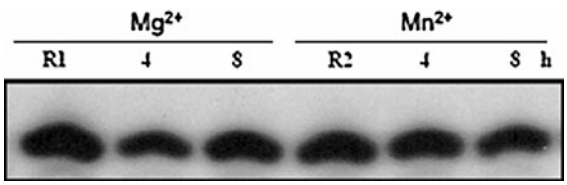

Fig. 5 Stability of the PS-oligo 3 in HP supplemented Mg^{2+} or Mn^{2+}ions. The 5′-labeled oligonucleotide (5 μM) was incubated in 50% HP in the presence of 10 mM Mg^{2+} or 10 mM Mn^{2+} at 37°C for 4 and 8 h. The resultant samples were analyzed by denaturing PAGE. *R1* and *R2* refer to the 5′-labeled samples (no HP added) dissolved in PBS containing 10 mM Mg^{2+} and 10 mM Mn^{2+} ions, respectively, and incubated at 37°C for 8 h

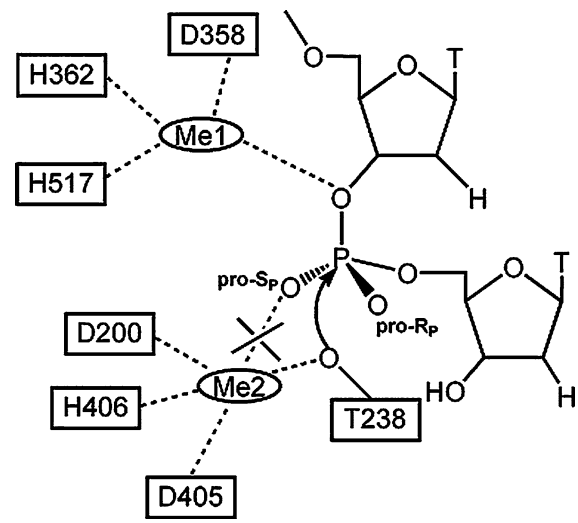

Fig. 6 Possible interactions between the single [S_P]-phosphorothioate linkage located at the 3′-end of oligonucleotide and metal ions (based on the model of Gijsbers et al. 2001)

an important functional link between chemical groups and metal ions. On the other hand, the knowledge about the metal specificity of the 3′-exonuclease is important for understanding the enzyme action under intracellular conditions in which Mg^{2+} is virtually the only divalent metal ion available at millimolar concentrations. In addtion, studies on the importance of stereochemistry of 3′-terminal phosphorothioate support an approach to protect oligonucleotides against 3′-exonucleolytic activity of certain enzymes based on the chirality of only the 3′-terminal phosphorothioate internucleotide linkage (Nawrot et al. 2008).

Taken together, a definitive determination of the role of divalent cations in the hydrolysis of PS-oligos catalyzed by the 3′-exonuclease will require the elucidation of its three-dimensional complex with a sulfur-substituted substrate DNA.

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References

Abaza MS, Al-Saffar A, Al-Sawan S, Al-Attiyah R (2008) c-myc antisense oligonucleotides sensitize human colorectal cancer cells to chemotherapeutic drugs. Tumour Biol 29:287–303. doi:10.1159/000156706

Bartlett PA, Eckstein F (1982) Stereochemical course of polymerization catalyzed by avian myeloblastosis virus reverse transcriptase. J Biol Chem 257:8879–8884

Belli SI, Sali A, Goding JW (1994) Divalent cations stabilize the conformation of plasma cell membrane glycoprotein PC-1 (alkaline phosphodiesterase I). Biochem J 304: 75–80

Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C (2000) Nucleotide pyrophosphatases/phosphodiesterases on the move. Crit Rev Biochem Mol Biol 35:393–432. doi:10.1080/10409230091169249

Bond MD, Holmquist B, Vallee BL (1986) Thioamide substrate probes of metal-substrate interactions in carboxypeptidase A catalysis. J Inorg Biochem 28:97–105

Brautigam CA, Steitz TA (1998) Structural principles for the inhibition of the 3′–5′ exonuclease activity of *Escherichia coli* DNA polymerase I by phosphorothioates. J Mol Biol 277:363–377. doi:10.1006/jmbi.1997.1586

Connolly BA, Eckstein F, Pingoud A (1984) The stereochemical course of the restriction endonuclease EcoRI-catalyzed reaction. J Biol Chem 259:10760–10763

Cowan JA (1998) Metal activation of enzymes in nucleic acid biochemistry. Chem Rev 98:1067–1087

Duan RD, Bergman T, Xu N, Wu J, Cheng Y, Duan J, Nelander S, Palmberg C, Nilsson A (2003) Identification of human intestinal alkaline sphingomyelinase as a novel ecto-enzyme related to the nucleotide phosphodiesterase family. J Biol Chem 278:38528–38536. doi:10.1074/jbc.M305437200

Eckstein F, Armstrong VW, Sternbach H (1976) Stereochemistry of polymerization by DNA-dependent RNA- polymerase from *Escherichia coli*: an investigation with a diastereomeric ATP-analogue. Proc Natl Acad Sci USA 73:2987–2990

Eckstein F, Sternbach H, von der Haar F (1977) Stereochemistry of internucleotidic bond formation by tRNA nucleotidyltransferase from baker′s yeast. Biochemistry 16:3429–3432

Eckstein F, Burgers PM, Hunneman DH (1979) Stereochemistry of hydrolysis by snake venom phosphodiesterase. J Biol Chem 254:7476–7478

Eder PS, DeVine RJ, Dagle JM, Walder JA (1991) Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3′-exonuclease in plasma. Antisense Res Dev 1:141–151

Frey PA, Sammons RD (1985) Bond order and charge localization in nucleoside phosphorothioates. Science 228: 541–545

Gijsbers R, Ceulemans H, Stalmans W, Bollen M (2001) Structural and catalytic similarities between nucleotide pyrophosphatases/phosphodiesterases and alkaline phosphatases. J Biol Chem 276:1361–1368. doi:10.1074/jbc.M007552200

Gilar M, Belenky A, Budman Y, Smisek DL, Cohen AS (1998) Impact of 3′-exonuclease stereoselectivity on the kinetics of phosphorothioate oligonucleotide metabolism. Antisense Nucleic Acids Drug Dev 8:35–42

Goding JW, Grobben B, Slegers H (2003) Physiological and pathophysiological functions of the ectonucleotide pyrophosphatase/phosphodiesterase family. Biochim Biophys Acta 1638:1–19. doi:10.1016/S0925-4439(03)00058-9

Grasby JA, Connolly BA (1992) Stereochemical outcome of the hydrolysis reaction catalyzed by the EcoRV restriction endonuclease. Biochemistry 31:7855–7861

Gupta AP, Benkovic SJ (1984) Stereochemical course of the 3′ → 5′ exonuclease activity of DNA polymerase. Biochemistry 23:5874–5881

Hoke GD, Draper K, Freier SM, Gonzalez C, Driver VB, Zounes MC, Ecker DJ (1991) Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. Nucleic Acids Res 19:5743–5748

Irwing H, Williams RJP (1953) The stability of transition metal complexes. J Chem Soc 3:3192–3210

Koziolkiewicz M, Wojcik M, Kobylanska A, Karwowski B, Rebowska B, Guga P, Stec WJ (1997) Stability of stereoregular oligo (nucleoside phosphorothioate)s in human plasma: diastereoselectivity of plasma 3′-exonuclease. Antisense Nucleic Acids Drug Dev 7:43–48

Koziolkiewicz M, Gendaszewska E, Maszewska M, Stein CA, Stec WJ (2001) The mononucleotide-dependent, nonantisense mechanism of action of phosphodiester and phosphorothioate oligonucleotides depends upon the

activity of an ecto-5′-nucleotidase. Blood 98:995–1002. doi:10.1182/blood.V98.4.995

Koziolkiewicz M, Owczarek A, Wojcik M, Domanski K, Guga P, Stec WJ (2002) Retention of configuration in the action of human plasma 3′-exonuclease on oligo (deoxynucleoside phosphorothioate). A new method for assignment of absolute configuration at phosphorus in isotopomeric deoxyadenosine 5′-O-[18O]-phosphorothioate. J Am Chem Soc 124:4623–4627. doi:10.1021/ja017187u

Luganini A, Caposio P, Landolfo S, Gribaudo G (2008) Phosphorothioate-modified oligodeoxynucleotides inhibit human cytomegalovirus replication by blocking virus entry. Antimicrob Agents Chemother 52:1111–1120. doi:10.1128/AAC.00987-07

Nawrot B, Paul N, Rebowska B, Stec WJ (2008) Significance of stereochemistry of 3′-terminal phosphorothioate-modified primer in DNA polymerase-mediated chain extension. Mol Biotechnol 40:119–126. doi:10.1007/s12033-008-9096-x

Oda Y, Kuo MD, Huang SS, Huang JS (1993) The major acidic fibroblast growth factor (aFGF)-stimulated phosphoprotein from bovine liver plasma membranes has aFGF-stimulated kinase, autoadenylylation, and alkaline nucleotide phosphodiesterase activities. J Biol Chem 268:27318–27326

Orr GA, Simon J, Jones SR, Chin GJ, Knowles JR (1978) Adenosine 5′-O-([gamma-18O]gamma-thio)triphosphate chiral at the gamma-phosphorus: stereochemical consequences of reactions catalyzed by pyruvate kinase, glycerol kinase, and hexokinase. Proc Natl Acad Sci USA 75:2230–2233

Pearson RG (1968) Hard and soft acids and bases, HSAB, part I: fundamental principles. J Chem Educ 45:581–587

Rebbe NF, Tong BD, Finley EM, Hickman S (1991) Identification of nucleotide pyrophosphatase/alkaline phosphodiesterase I activity associated with the mouse plasma cell differentiation antigen PC-1. Proc Natl Acad Sci USA 88:5192–5196

Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y, Arai H (2005) Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. J Biol Chem 280:23084–23093. doi:10.1074/jbc.M413438200

Sheu KF, Frey PA (1978) UDP-glucose pyrophosphorylase. Stereochemical course of the reaction of glucose 1-phosphate with uridine-5′ [1-thiotriphosphate]. J Biol Chem 253:3378–3380

Stec WJ, Grajkowski A, Kobylanska A, Karwowski B, Koziolkiewicz M, Misiura K, Okruszek A, Wilk A, Guga P, Boczkowska M (1995) Diastereomers of nucleoside 3-O-2-thio-1.3.2 oxathia(selena)phospholanes): building blocks for stereocontrolled synthesis of oligo(nucleoside phosphorothioate)s. J Am Chem Soc 117:12020–12029

Stefan C, Jansen S, Bollen M (2005) NPP-type ecto-phosphodiesterases: unity in diversity. Trends Biochem Sci 30:542–550. doi:10.1016/j.tibs.2005.08.005

Stewart DJ, Donehower RC, Eisenhauer EA, Wainman N, Shah AK, Bonfils C, MacLeod AR, Besterman JM, Reid GK (2003) A phase I pharmacokinetic and pharmacodynamic study of the DNA methyltransferase 1 inhibitor MG98 administered twice weekly. Ann Oncol 14:766–774. doi:10.1093/annonc/mdg216

Vaerman JL, Moureau P, Deldime F, Lewalle P, Lammineur C, Morschhauser F, Martiat P (1997) Antisense oligodeoxyribonucleotides suppress hematologic cell growth through stepwise release of deoxyribonucleotides. Blood 90:331–339

Wang S, Karbstein K, Peracchi A, Beigelman L, Herschlag D (1999) Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate. Biochemistry 38:14363–14378. doi:10.1021/bi9913202

Warnecke JM, Furste JP, Hardt WD, Erdmann VA, Hartmann RK (1996) Ribonuclease P (RNase P) RNA is converted to a Cd (2+)-ribozyme by a single R_P-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site. Proc Natl Acad Sci USA 93:8924–8928

Wojcik M, Cieslak M, Stec WJ, Goding JW, Koziolkiewicz M (2007) Nucleotide pyrophosphatase/phosphodiesterase 1 is responsible for degradation of antisense phosphorothioate oligonucleotides. Oligonucleotides 17:134–145. doi:10.1089/oli.2007.0021

Zhou DM, He QC, Zhou JM, Taira K (1998) Explanation by a putative triester-like mechanism for the thio effects and Mn^{2+} rescues in reactions catalyzed by a hammerhead ribozyme. FEBS Lett 431:154–160. doi:10.1016/S0014-5793(98)00734-0

Zon G, Stec WJ (1991) Phosphorothioate oligonucleotides. In: Eckstein F (ed) Oligonucleotides and analogues: a practical approach. IRL Press, Oxford, pp 87–108