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Effect of different N7 substitution of dinucleotide cap analogs on the hydrolytic susceptibility towards scavenger decapping enzymes (DcpS)

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ABSTRACT

Scavenger decapping enzymes (DcpS) are involved in eukaryotic mRNA degradation process. They catalyze the cleavage of residual cap structure m^7GpppN and/or short capped oligonucleotides resulting from exosom–mediated the 3′ to 5′ digestion. For the specific cap recognition and efficient degradation by DcpS, the positive charge at N7 position of guanine moiety is required. Here we examine the role the N7 substitution within the cap structure on the interactions with DcpS (human, *Caenorhabditis elegans* and *Ascaris suum*) comparing the hydrolysis rates of dinucleotide cap analogs (m^7GpppG, et^{7}GpppG, but^{7}GpppG, bn^{7}GpppG) and the binding affinities of hydrolysis products (m^7GMP, et^{7}GMP, but^{7}GMP, bn^{7}GMP). Our results show the conformational flexibility of the region within DcpS cap-binding pocket involved in the interaction with N7 substituted guanine, which enables accommodation of substrates with differently sized N7 substituents.

1. Introduction

Eukaryotic mRNAs are modified at the 5′ end with a cap structure (m^7GpppN, where N = A,G,C or U) consisting of 7-methylguanosine attached to the first transcribed nucleotide of mRNA chain through an unusual 5′-5′ pyrophosphate bond [1,2]. This terminal distinctive mark plays an essential role in the post-transcriptional control of gene expression. It allows to recognize mRNA 5′ end by specific proteins involved in various cellular processes, including intracellular transport, translation and decay [3]. In the nucleus, cap interacts with CBC complex participating in the nuclear export [4]. In the cytoplasm, cap recognition by eIF4E is a rate-limiting step in translation initiation [5]. In mRNA degradation, the cap is bound and hydrolyzed by specific pyrophosphatases: DcpS, Dcp2, Nudt16 [6–8].

mRNA degradation is a tightly regulated process which eliminates defective transcripts from the cells and allows adaptation to environmental changes. The decay of mRNA occurs *via* two general pathways, in 5′ → 3′ or 3′ → 5′ direction, both initiated by the shortening of the poly(A) tail [6,9]. In the 5′ → 3′ decay, deadenylated mRNAs are subject to decapping enzyme Dcp2 generating m^7GDP and 5′ phosphorylated RNA chain. The 3′ → 5′ decay is carried out by a cytoplasmic multisubunit exosome complex and the resulting cap dinucleotide (or short oligonucleotide) is subsequently hydrolyzed by the scavenger decapping enzyme (DcpS) [6,10]. Hydrolysis of the cap structure releases m^7GMP and ppN or

Abbreviations: A. suum., Ascaris suum; *C. elegans*, *Caenorhabditis elegans*; CBC, cap-binding complex; DcpS, decapping scavenger; DTT, dithiotreitol; eIF4E, eukaryotic initiation factor 4E; HIT, histidine triad; HPLC, high performance liquid chromatography.

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diphosphate terminated oligonucleotides shorter than 10 nucleotides [6,10].

Decapping scavengers have been identified in various species, from yeast to mammals [11–13]. However, the hydrolytic activity and binding affinity have been well characterized for human and *Caenorhabditis elegans* DcpS, indicating that these two enzymes differ in substrate length and capacity to hydrolyze trimethyleted cap analogs [14,15]. For the specific cap recognition and efficient degradation by DcpS, the following structural features are required: (1) the positive charge at the N7 position of guanine moiety, (2) 2′OH and 3′OH groups in the ribose ring of 7-methylguanosine, (3) at least triphosphate groups in the phosphate bridge [15]. Various cap analogs modified within pyrophosphate bridge, ribose ring of 7-methylguanosine and the second nucleoside were synthesized to study their binding affinities and hydrolytic susceptibilities towards decapping scavengers [16–18]. It was shown that modifications within the phosphate chain affects binding affinity depending on the type and the position of a substituent, and the methylation of 2′OH or 3′OH group in the ribose ring of 7-methylguanosine significantly decreases the binding affinity and hydrolysis [15].

The impact of N7 substitution has not been investigated so far. The initial kinetic studies performed for recombinant *C. elegans* DcpS indicate that this enzyme accepts as substrates dinucleotides with differently sized substituents (methyl, ethyl and benzyl) at N7 position [17]. To further investigate the effect of N7 substitution, we performed the comparative studies on the influence of alkyl or benzyl groups introduced at N7 position of guanine moiety of cap analogs on their substrate properties towards decapping scavengers from different species: human, *C. elegans* and *A. suum*. We examined hydrolytic susceptibility of dinucleotide cap analogs: m^7GpppG (7-methylGpppG), et^7GpppG (7-ethylGpppG), but^7GpppG (7-butylGpppG), bn^7GpppG (7-benzylGpppG) and binding affinities of their mononucleotide counterparts m^7GMP, et^7GMP, but^7GMP, bn^7GMP.

2. Materials and methods

2.1. Materials

Cap analogs investigated in this work were synthesized according to the methods described earlier [19–21]. Decapping scavengers: human DcpS (*Hs*DcpS), *C. elegans* DcpS (*Ce*DcpS) and *A. suum* DcpS (*As*DcpS) were expressed in *Escherichia coli* and purified as His-tagged proteins by affinity chromatography using Ni-NTA agarose under native conditions. To obtain homogeneous fractions of DcpS, recombinant enzymes were further purified by gel filtration through a Pharmacia Superdex-200 column (GE Healthcare Bioscience AB) connected to AKTA FPLC system (Pharmacia-Biotech) [14]. The concentration of cap analogs and DcpS enzymes were estimated from their molar absorption coefficients [14,22].

2.2. Enzymatic assays

The hydrolytic activity of recombinant DcpS was assayed in 50 mM phosphate buffer pH 7.2 containing 150 mM NaCl and 1 mM DTT, at 20 °C. Initial cap concentration for DcpS-mediated hydrolysis was 20 μM. Before each experiment, 1 mL of buffer solution containing the investigated compound was incubated at 20 °C for 10 min. The hydrolysis process was initiated by the addition of recombinant DcpS. After 5, 10, 15 and 20 min of the hydrolysis, 150 μL aliquots of the reaction mixture were withdrawn and incubated at 97 °C during 3 min to stop the reaction by heat inactivation of the enzyme. The samples were then subjected to analytical HPLC (Agilent 1200 Series) equipped with a reverse-phase Supelcosil LC-18-T column and UV/VIS detector. Substrate and products were eluted at room temperature with a linear gradient of methanol in 0.1 M KH_2PO_4 (from 0% to 25% for m^7GpppG and et^7GpppG, from 0% to 40% for but^7GpppG, from 0% to 50% for bn^7GpppG) over 15 min at a flow rate of 1.3 mL/min. The changes of absorbance at 260 nm were monitored continuously during the analysis. Hydrolysis products were identified by comparison of their retention times with the reference samples. The extent of decapping determined as the percentage of hydrolyzed substrate was calculated from the area under the chromatographic peak of respective dinucleotide.

2.3. Determination of association constants by fluorescence titration experiments

DcpS binding affinities for mononucleotide cap analogs were determined by monitoring the quenching of intrinsic Trp fluorescence of the proteins. The experiments were performed on LS-55 spectrofluorometer (Perkin–Elmer) in a quartz cuvette (Hellma) with an optical path length of 4 mm for absorption and 10 mm for emission. All measurements were performed at 20 °C, in 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 1 mM DTT. Aliquots of 1 μL of respective cap analog solution of increasing concentration (from 2 μM to 1 mM) were added to 1 mL of DcpS solution (initial concentration 0.2 μM). The fluorescence intensity was monitored at 340 nm with a 4–5 nm bandwidth (excitation at 280 nm with a 5 nm bandwidth) and corrected for sample dilution and inner filter effects. The equilibrium association constants for single titrations (K_{as}) were determined by fitting the theoretical dependence of the fluorescence intensity on the total concentration of the cap analog to the experimental data points using the previously described equation [16]. The final K_{as} were calculated as weighted averages of 3 independent titrations, with the weights taken as the reciprocal standard deviations squared. The numerical least-squares non-linear regression analysis was performed using ORIGIN 9.0.

3. Results

3.1. Comparison of hydrolytic susceptibility of dinucleotide cap analogs modified at N7 position

The influence of N7 substitution on the hydrolytic susceptibility of dinucleotide cap analogs to decapping scavengers has been examined by comparison the hydrolysis efficiency of et^7GpppG, but^7GpppG and bn^7GpppG with natural substrate m^7GpppG. As presented in Fig. 1, all investigated dinucleotides are hydrolyzed by human and both nematode DcpS. However, small differences are observed in their substrate properties towards human and nematode DcpS. The comparison of the kinetic results indicates that introduction of a bulkier substituent at N7 position significantly increases the hydrolytic susceptibility of dinucleotide cap analogs for nematode decapping scavengers. For *Ce*DcpS, but^7GpppG occurs to be the most efficiently hydrolyzed substrate, about 2.5-times faster than m^7GpppG. For *As*DcpS, the hydrolysis rate increase about 2-times for et^7GpppG, but^7GpppG and bn^7GpppG. Different effect is observed in the case of *Hs*DcpS, for which the natural substrate m^7GpppG is hydrolyzed more efficient in comparison with et^7GpppG, but^7GpppG and bn^7GpppG. The results show that decapping scavengers from different species accept as substrates dinucleotides with differently sized substituents (ethyl, butyl, benzyl) instead of methyl group at N7 position. The accommodation of substrates with bulkier N7 substituent indicate the important role of the conformational

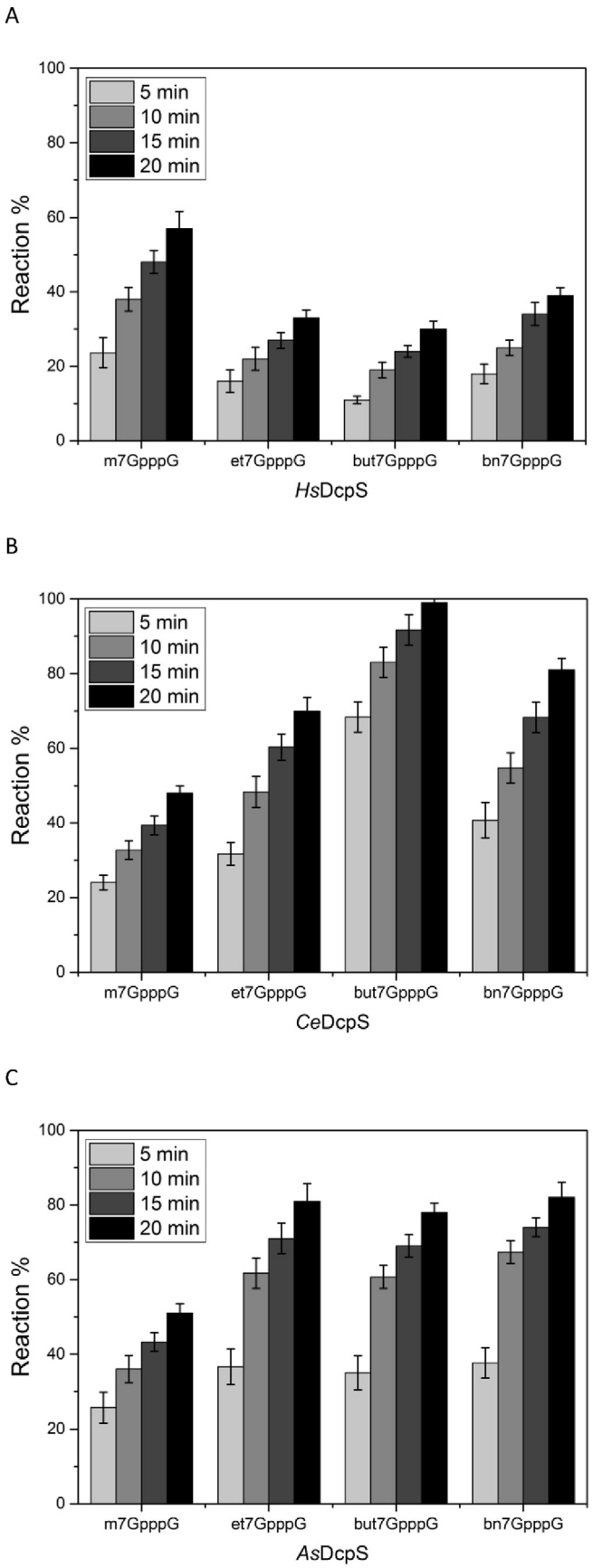

Fig. 1. Comparison of hydrolytic susceptibility of m^7GpppG, et^7GpppG, but^7GpppG, bn^7GpppG towards decapping scavengers: *Hs*DcpS (A), *Ce*DcpS (B) and *As*DcpS (C). The extent of decapping was determined as the % of hydrolyzed substrate measured by HPLC system (based on the 3 independent experiments for each compound). All experiments were performed at 20 °C in 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl. The following enzyme concentration in reaction mixture were used: 0.03 μM *Hs*DcpS, 0.02 μM *Ce*DcpS and 0.01 μM *As*DcpS. Initial concentration of cap analogs was 20 μM.

flexibility of the region within cap-binding pocket of DcpS, which is involved in the interaction with N7 substituted guanine and complex formation.

3.2. Binding affinity studies of mononucleotide cap analogs modified at N7 position

To further determine the role of the N7 substituents in the interactions with DcpS enzymes, we compared the binding affinities of hydrolysis products: m^7GMP, et^7GMP, but^7GMP, bn^7GMP. The association constants (K_{as}) for the complexes of decapping scavengers with N7 substituted mononucleotides were calculated on the basis of fluorescence titration curves (Fig. 2) and summarized in Table 1. For *Hs*DcpS and *As*DcpS, the highest K_{as} values are observed with m^7GMP. The binding affinity with m^7GMP is comparable for these two enzymes, whereas for *C. elegans* DcpS is about 2-times lower. The presence of ethyl, butyl or benzyl at N7 position significantly changes the strength of interaction with decapping scavengers. The effect of different type of N7 substitution is not the same for the three enzymes. Human DcpS exhibit near the same affinity for m^7GMP and but^7GMP but significantly lower binding is observed for et^7GMP and bn^7GMP. *As*DcpS efficiently binds only m^7GMP. Association constants for et^7GMP, but^7GMP, bn^7GMP are lower about one order of magnitude. Small decrease of binding affinity is observed for *C. elegans* DcpS, when methyl group is replaced by butyl or benzyl, whereas the presence of ethyl does not affect binding. In general, the mononucleotides with bulkier substituents at N7 position of 7-methylguanosine are bound less efficiently by DcpS enzymes. Weaker binding of the reaction products makes easier their release from complexes with nematode DcpS during catalytic cycle, increasing the hydrolysis rate. The effect of bulkier substituent at N7 position is different, when respective dinucleotides are hydrolyzed by human DcpS. et^7GMP, but^7GMP and bn^7GMP occurred to be less efficiently degraded by this enzyme.

4. Discussion

DcpS enzymes are members of the HIT family of pyrophosphatases containing a conserved histidine triad (His-X-His-X-His) in the active site [23–25]. The central histidine within the HIT motif plays an important role as a nucleophilic agent in the hydrolysis process [26]. The positively charged 7-methylguanine moiety is the main structural determinant for cap analogs recognition by DcpS enzymes. The crucial role of this specific feature is reflected by efficient hydrolysis of m^7GpppG, catalyzed by human and nematode decapping scavengers [14], in contrast to non-methylated dinucleotide GpppG, which is resistant to *Hs*DcpS and *As*DcpS [17].

Presented here kinetic experiments and binding affinity studies with various cap analogs bearing a bulkier substituent at N7 position of 7-methylguanosine (ethyl, butyl or benzyl) revealed that the nucleobase-binding site of DcpS enzymes is inherently flexible. This flexibility explains why dinucleotides with differently sized groups at N7 position are capable to bind and being hydrolyzed, even more efficiently in comparison with the natural substrate m^7GpppG, as it was observed for nematode decapping scavenger (Fig. 1B, C). Crystallographic data and docking analysis of DcpS binding site

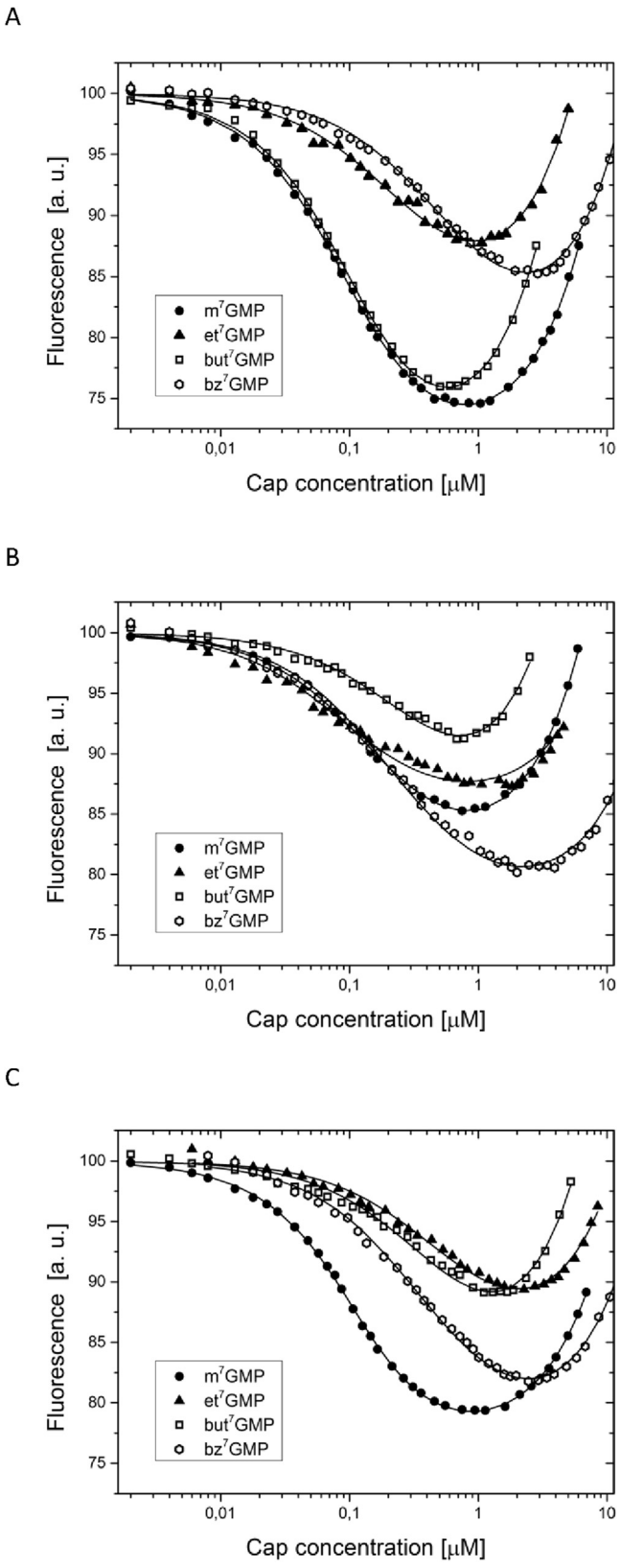

Fig. 2. Fluorescence titration curves for binding of mononucleotide cap analogs (m^7GMP, et^7GMP, but^7GMP, bn^7GM) to *Hs*DcpS (A), *Ce*DcpS (B) and *As*DcpS (C). Titration experiments were performed at 20 °C in 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl. DcpS fluorescence was excited at 280 nm and observed at 340 nm. The increase of fluorescence signal observed at higher concentrations of cap analogs originates from the fluorescence of free cap molecules in the solution. The intensity of fluorescence is presented as relative values (AU: arbitrary units).

Table 1
Equilibrium association constants (K_{as}) for the complexes of mononucleotide cap analogs with the human and nematode DcpS, obtained from analysis of steady-state fluorescence titration at 20 °C.

Cap analog	K_{as} [μM^{-1}]		
	*Hs*DcpS	*Ce*DcpS	*As*DcpS
m^7GMP	16.22 ± 0.61	7.19 ± 0.38	18.11 ± 0.54
et^7GMP	4.07 ± 0.95	9.12 ± 2.17	2.05 ± 0.32
but^7GMP	12.85 ± 0.93	3.37 ± 0.75	2.10 ± 0.40
bn^7GMP	1.74 ± 0.19	4.27 ± 0.31	2.55 ± 0.23

[15,24,25] indicate that nucleobase of 7-methylguanosine moiety makes stacking interactions with the highly conserved tryptophan residues (W175 for *Hs*DcpS, W149 for *Ce*DcpS and W144 for *As*DcpS) and hydrophobic van der Waals contacts with the aliphatic side chain (L206 for *Hs*DcpS, I180 for *Ce*DcpS and L175 for *As*DcpS). The orientation of 7-methylguanine is enforced by a highly hydrophobic cluster formed by amino acids interacting with this part of cap analogs. The hydrophobicity and flexibility of the region involved in stacking interactions enables to accommodate cap structures with ethyl, butyl or benzyl at N7 position.

The hydrolysis rate depends on DcpS interactions with substrate molecules and the rate of product release. As presented in this study, the lower binding affinities of et^7GMP, but^7GMP and bn^7GMP with nematode DcpS are observed, compared to m^7GMP (Table 1). As a result, et^7GpppG, but^7GpppG and bn^7GpppG are hydrolyzed by these enzymes more efficiently than the natural substrate m^7GpppG. Interestingly, in the case of *Hs*DcpS, the presence of a bulkier substituent at N7 position of cap analogs decreases their hydrolysis rate, despite of weaker binding determined for et^7GMP, but^7GMP and bn^7GMP. This effect may be explain by different conformation of N7 substituted dinucleotides in the cap binding pocket of human and nematode DcpS or influence of product inhibition.

The importance of interactions of N7-alkylated and N7-benzylated monophosphates has been also recently studied with another cap-binding protein, eIF4E [27,28]. On the basis of crystallographic structure and molecular docking it was demonstrated that the N7 benzyl groups pack into a hydrophobic pocket behind the two tryptophan groups involved in cap recognition, similar as in DcpS proteins. It was shown, that benzyl substitution increased 3-fold the binding affinity with eIF4E in comparison with m^7GMP. Both cap-binding proteins, DcpS and eIF4E exhibit flexibility in the 7-methylguanine binding site which enables accommodation of larger substituents. N7-benzylated cap analogs have been proposed as new families of eIF4E inhibitors with potential therapeutic applications in cancer [29]. Therapeutic activity of cap analogs depends on their stability in cellular conditions and DcpS enzymes can effectively compete for cap binding with eIF4E. As N7-alkylated and N7-benzylated cap analogs are recognized by both proteins, DcpS enzymes may influence the interaction with eIF4E.

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