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Structural requirements for *Caenorhabditis elegans* DcpS substrates based on fluorescence and HPLC enzyme kinetic studies

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The activity of the *Caenorhabditis elegans* scavenger decapping enzyme (DcpS) on its natural substrates and dinucleotide cap analogs, modified with regard to the nucleoside base or ribose moiety, has been examined. All tested dinucleotides were specifically cleaved between β- and γ-phosphate groups in the triphosphate chain. The kinetic parameters of enzymatic hydrolysis (K_m, V_{max}) were determined using fluorescence and HPLC methods, as complementary approaches for the kinetic studies of *C. elegans* DcpS. From the kinetic data, we determined which parts of the cap structure are crucial for DcpS binding and hydrolysis. We showed that $m_3^{2,2,7}$GpppG and $m_3^{2,2,7}$GpppA are cleaved with higher rates than their monomethylated counterparts. However, the higher specificity of *C. elegans* DcpS for monomethylguanosine caps is illustrated by the lower K_m values. Modifications of the first transcribed nucleotide did not affect the activity, regardless of the type of purine base. Our findings suggest *C. elegans* DcpS flexibility in the first transcribed nucleoside-binding pocket. Moreover, although *C. elegans* DcpS accommodates bulkier groups in the N7 position (ethyl or benzyl) of the cap, both 2′-*O*- and 3′-*O*-methylations of 7-methylguanosine result in a reduction in hydrolysis by two orders of magnitude.

Introduction

mRNA turnover is a critical determinant in the regulation of gene expression [1–3]. The degradation of normal transcripts in eukaryotes occurs along two major pathways, 5′ → 3′ and 3′ → 5′ decay, both initiated by shortening of the poly(A) tail [4,5]. In the 5′ → 3′ decay pathway, deadenylation is followed by Dcp1/Dcp2-mediated decapping, which exposes the body of the transcript to Xrn1 exonuclease [6,7]. In the 3′ → 5′ decay pathway, deadenylation facilitates access to the mRNA 3′ end by a complex of nucleases, known as the exosome, which degrades the mRNA chain 3′ → 5′ until it reaches the cap-containing dinucleotide or a short capped oligonucleotide [8,9]. The residual cap structure m^7GpppN (7-methylGpppN) is further hydrolyzed by the scavenger decapping enzyme (DcpS) [10]. Capped dinucleotides or oligonucleotides accumulated in cells could bind to cap-binding proteins, such as eIF4E, and inhibit translation [11]. The hydrolysis of cap dinucleotides in this context is thought to be important. However,

Abbreviations
ARCA (anti-reverse cap analog), $m_2^{7,2'\text{-}O}$GpppG and $m_2^{7,3'\text{-}O}$GpppG; bn^{7}GpppG, 7-benzylGpppG; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; DcpS, scavenger decapping enzyme; et^{7}GpppG, 7-ethylGpppG; HIT, histidine triad; $m_3^{2,2,7}$GpppG, trimethylguanosine cap; m^7GpppN, 7-methylGpppN; m^7Guo, 7-methylguanosine; MMG and TMG cap, monomethylguanosine and trimethylguanosine cap.

mutations in DcpS are generally not lethal, suggesting the possibility that other undiscovered and redundant scavenger enzyme activities may be present [11,12].

Decapping scavengers have been characterized in yeast (*Saccharomyces cerevisiae* and *Saccharomyces pombe*), nematode (*Caenorhabditis elegans* and *Ascaris suum*) and mammalian (mouse and human) cells [13–15]. DcpS proteins constitute their own branch within the histidine triad (HIT) family of pyrophosphatases, with decapping activity as the main, well-defined biological function [16,17]. All of these enzymes exhibit high specificity for cap structure and limited activity towards nonmethylated dinucleotides (e.g. ApppA and GpppG). Decapping scavengers utilize an evolutionary conserved HIT motif to cleave the 5′-ppp-5′ pyrophosphate bond within the cap, releasing m^7GMP [15–17]. Sequence alignment of DcpS proteins from different organisms demonstrated the presence of a conserved hexapeptide containing HIT with three histidines separated by hydrophobic residues (His-φ-His-φ-His-φ). Structural analysis has revealed that HIT proteins exist as homodimers containing nucleotide-binding pockets with respect to the three histidine residues of the catalytic HIT motif [18–20]. A high degree of identity observed in the HIT region of different scavengers supports the functional significance of this domain in decapping activity. Substitution mutagenesis of the central histidine in human and nematode decapping scavengers inactivates their hydrolytic properties, demonstrating that the central HIT motif is critical for catalysis [14,20]. This histidine is involved in the formation of a covalent nucleotidyl phosphohistidyl intermediate, the nucleophilic agent for the γ-phosphate group of dinucleoside triphosphate substrates [19,20].

The process of mRNA turnover is more complicated in nematodes, because they have two populations of mRNAs, each with a distinct cap structure. Approximately 70% of nematode mRNAs possess a trimethylguanosine cap ($m_3^{2,2,7}$GpppG), whereas approximately 30% have a typical cap structure (m^7GpppG) [21]. Both types of mRNA interact with polysomes and undergo translation [12,22]. The presence of two populations of mRNAs has profound implications for proteins that recognize specifically each mRNA [23]. The eIF4E protein in *C. elegans* exists in five different isoforms, with different affinity to m^7GpppG and $m_3^{2,2,7}$GpppG [20,21]. Human and yeast DcpS can effectively hydrolyze only the m^7GpppG cap, and human DcpS has activity on capped oligonucleotides up to 10 nucleotides [22–24]. In contrast, initial studies on the nematode decapping scavenger indicated that both trimethylated and monomethylated caps and oligonucleotides up to four nucleotides were hydrolyzed [14].

Previous data have suggested that the substrate specificity of *C. elegans* DcpS differs from that of its human and yeast orthologs [3,14,25,26]. However, neither detailed kinetic analysis of enzymatic cleavage nor mechanisms of substrate recognition have been investigated on *C. elegans* DcpS. In this article, we have studied the substrate specificity and kinetic analysis of recombinant *C. elegans* DcpS. Various dinucleotide cap analogs, natural and chemically modified within the 7-methylgunosine moiety or the first transcribed nucleoside, have been investigated as potential substrates. Kinetic parameters (K_m, V_{max} and V_{max}/K_m) were determined to characterize the hydrolytic activity of *C. elegans* DcpS.

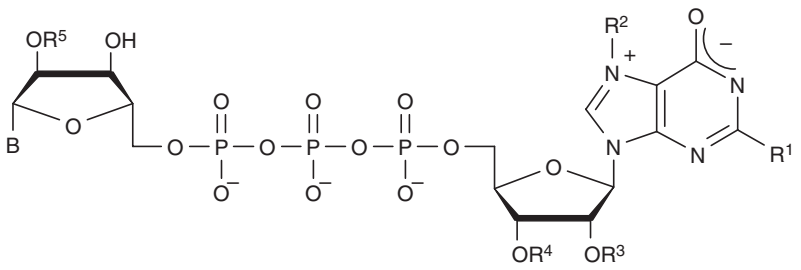
Results

Decapping products of reactions catalyzed by *C. elegans* DcpS

To identify the DcpS hydrolysis products of all investigated dinucleotides presented in Fig. 1, high-performance liquid chromatograms were analyzed. As an example, chromatographic analysis for the cleavage of monomethylguanosine (MMG) cap, trimethylguanosine (TMG) cap and GpppG are shown in Fig. 2. For $m_3^{2,2,7}$GpppG, the peak corresponding to the substrate disappeared after 10 min of reaction (Fig. 2A). MMG was almost completely hydrolyzed over 20 min (Fig. 2B). The hydrolysis of GpppG was much slower – after 120 min a considerable amount of the substrate was still observed in the reaction mixture (Fig. 2C). The analysis of the hydrolysis products (Table 1) demonstrates that the cleavage of cap analogs occurs exclusively between β- and γ-phosphate groups within the triphosphate bridge. These data confirm the earlier observations that nematode DcpS utilizes the same mechanism of catalysis as proposed for other HIT pyrophosphatases cleaving the cap structure, and the highly conserved HIT motif is involved in the binding of the substrates and catalysis [19,20].

Specificity of *C. elegans* DcpS towards MMG and TMG caps

Initial studies on the substrate specificity of recombinant *C. elegans* DcpS suggested that the protein was specific for 7-methylguanosine (m^7Guo) nucleotides. The first quantitative experiments characterizing this enzyme were reported by Kwasnicka *et al.* [25]. However, the specificity of *C. elegans* DcpS was defined



Cap analogue	Reference to synthesis	Structure
m^7GpppG	33	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = guanine
$m_3^{2,2,7}GpppG$	33	$R^1 = N(CH_3)_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = guanine
m^7GpppA	33	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = adenine
$m_3^{2,2,7}GpppA$	33	$R^1 = N(CH_3)_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = adenine
$m_2^{7,2'\text{-}O}GpppG$	28	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = CH_3$, $R^4 = H$, $R^5 = OH$, B = guanine
$m_2^{7,3'\text{-}O}GpppG$	27	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = H$, $R^4 = CH_3$, $R^5 = OH$, B = guanine
bn^7GpppG	38	$R^1 = NH_2$, $R^2 = CH_2C_6H_5$, $R^3 = R^4 = H$, $R^5 = OH$, B = guanine
et^7GpppG	38	$R^1 = NH_2$, $R^2 = CH_2CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = guanine
m^7Gpppm^7G	34	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = 7-methyl-guanine
$m^7Gppp2'dG$	35	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = R^5 = H$, B = guanine
$m^7Gpppm^{2'\text{-}O}G$	35	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OCH_3$, B = guanine
m^7Gpppm^6A	35	$R^1 = NH_2$, $R^2 = CH_3$, $R^3 = R^4 = H$, $R^5 = OH$, B = N^6-methyl-adenine

Fig. 1. Structures of the investigated cap analogs and references to their synthesis.

with $m^7GpppBODIPY$, GpppBODIPY and ApppBODIPY (BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene), but not with natural caps m^7GpppG or $m_3^{2,2,7}GpppG$. Methylated mono- and dinucleotides (m^7GDP, m^7GTP, m^7GpppG, $m_3^{2,2,7}GpppG$) have only been examined as inhibitors of *C. elegans* scavenger in the hydrolysis process of $m^7GpppBODIPY$. The inhibition constant calculated for $m_3^{2,2,7}GpppG$ (K_i = 28.1 ± 2.5 μM), eight-fold higher than for m^7GpppG (K_i = 3.47 ± 0.84 μM), indicated less efficient inhibitory properties of the trimethylated cap in comparison with its monomethylated counterpart. On the basis of these findings, it was concluded that the TMG cap may not be a substrate for *C. elegans* DcpS. In subsequent studies, both MMG and TMG caps were shown to be hydrolyzed by *C. elegans* scavenger (cellular extract and recombinant protein), but the substrate affinity and kinetics of this reaction with the substrates were not determined quantitatively [14]. To make a detailed comparison of *C. elegans* DcpS activity for the natural mono- and trimethylated caps, we carried out kinetic studies of hydrolysis of m^7GpppG, m^7GpppA, $m_3^{2,2,7}GpppG$ and $m_3^{2,2,7}GpppA$ using a fluorimetric method. The Michaelis–Menten curves (v_o versus c_o) obtained for these compounds are presented in Fig. 3.

The initial velocity data showed that the kinetics for MMG and TMG caps were hyperbolic in the investigated concentration ranges: 0.5–86 μM for m^7GpppG and 0.5–97 μM for $m_3^{2,2,7}GpppG$. The kinetic parameters derived for these reactions, Michaelis constants (K_m), maximum velocities (V_{max}) and pseudo-first-order rate constants (V_{max}/K_m) are summarized in Table 1. The K_m and V_{max} values are about three times higher for the TMG cap than for the MMG cap, whereas the V_{max}/K_m values are almost the same. This indicates that *C. elegans* DcpS has slightly different substrate specificities for these natural compounds,

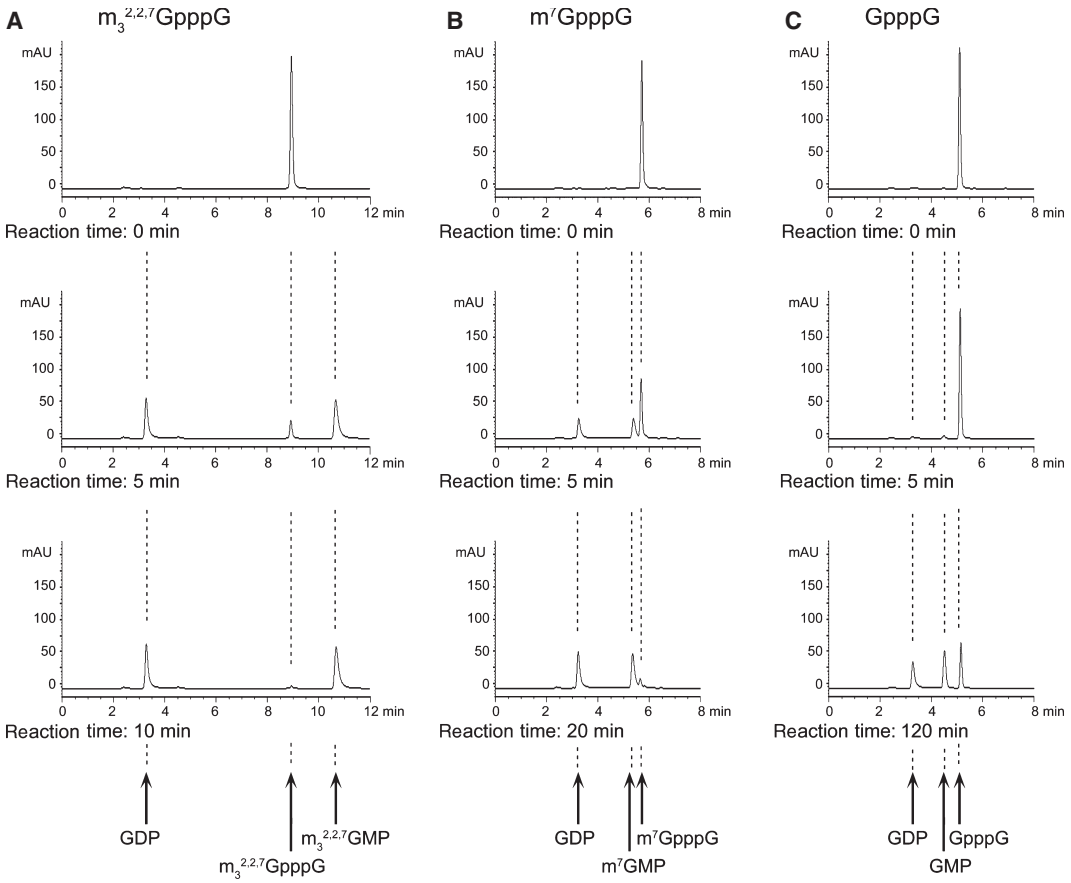


Fig. 2. HPLC profiles for the hydrolysis of $m_3^{2,2,7}$GpppG (A), m^7GpppG (B) and GpppG (C) catalyzed by *Caenorhabditis elegans* DcpS. The initial concentration of each substrate was 10 μM and the reactions were carried out with the same amount of enzyme: 1 μg. Absorbance was measured at 260 nm (AU, arbitrary units). The chromatographic peaks were identified by comparison with the retention times of reference samples.

with a preference for m^7GpppG, as suggested previously [25,26]. However, the rate of hydrolysis catalyzed by *C. elegans* DcpS is higher for the TMG cap.

Kinetics of cap analogs modified in the first transcribed nucleoside

To further examine the substrate specificity of *C. elegans* DcpS, the hydrolysis of several other dinucleotide cap analogs was examined. Substitution of adenine for guanine as the second nucleotide in MMG and TMG caps did not change significantly the substrate properties of m^7GpppA and $m_3^{2,2,7}$GpppA for DcpS catalysis when compared with m^7GpppG and $m_3^{2,2,7}$GpppG, respectively (Table 1). Similarly, monomethylated cap dinucleotides of the type m^7GpppN, modified within the first transcribed nucleoside (N = m^6A, m^7G, 2′dG, $m^{2'\text{-}O}$G) were all good DcpS substrates, as illustrated by the kinetic data (Fig. 3, Table 1). The K_m and V_{max} values for these four compounds are similar to that obtained for the MMG cap, indicating that C. *elegans* DcpS tolerates different modifications within the first transcribed nucleoside. The data presented here show that the second nucleotide of the cap structure is not crucial for the catalytic mechanism of *C. elegans* DcpS.

Kinetics of cap analogs modified in m^7Guo

The next interesting part of our studies concerning the substrate requirements for *C. elegans* DcpS revealed that the enzyme tolerates differently sized substituents at the N7 position of m^7Guo. The kinetic data (K_m, V_{max} and V_{max}/K_m) calculated for m^7GpppG (7-methyl GpppG), et^7GpppG (7-ethylGpppG) and bn^7GpppG (7-benzylGpppG) clearly showed that all three compounds are similarly recognized as substrates by the nematode scavenger (Table 1). These findings suggest

Table 1. Comparison of the substrate specificity of cap analogs towards *Caenorhabditis elegans* DcpS, obtained by the initial velocity method at 20 °C in 50 mM Tris/HCl buffer containing 30 mM $(NH_4)_2SO_4$ and 20 mM $MgCl_2$ (pH 7.2).

Cap analog	Products of hydrolysis	K_m (μM)	V_{max} (U·mg^{-1})	V_{max}/K_m (min^{-1}·mg^{-1})
Fluorescence method				
m^7GpppG	m^7GMP + GDP	1.17 ± 0.14	1.53 ± 0.11	1.30 ± 0.18
m^7GpppA	m^7GMP + ADP	0.60 ± 0.11	1.09 ± 0.11	1.83 ± 0.38
m^7Gpppm^{6}A	m^7GMP + m^6ADP	1.03 ± 0.16	1.33 ± 0.12	1.30 ± 0.23
m^7Gpppm^{7}G	m^7GMP + m^7GDP	1.12 ± 0.14	0.91 ± 0.10	0.81 ± 0.13
m^7Gpppm$^{2'\text{-}O}$G	m^7GMP + $m^{2'\text{-}O}$GDP	1.23 ± 0.13	1.66 ± 0.12	1.35 ± 0.17
m^7Gppp2′dG	m^7GMP + 2′dGDP	1.36 ± 0.41	2.00 ± 0.26	1.47 ± 0.48
$m_2^{7,2'\text{-}O}$GpppG	$m_2^{7,2'\text{-}O}$GMP + GDP	42.13 ± 3.91	3.28 ± 0.19	0.08 ± 0.01
$m_2^{7,3'\text{-}O}$GpppG	$m_2^{7,3'\text{-}O}$GMP + GDP	15.39 ± 2.08	0.51 ± 0.11	0.03 ± 0.01
et^{7}GpppG	et^{7}GMP + GDP	0.61 ± 0.18	3.12 ± 1.45	5.09 ± 2.80
bn^{7}GpppG	bn^{7}GMP + GDP	1.83 ± 0.15	3.06 ± 0.12	1.67 ± 0.15
$m_3^{2,2,7}$GpppG	$m_3^{2,2,7}$GMP + GDP	3.85 ± 0.41	4.65 ± 0.26	1.21 ± 0.15
$m_3^{2,2,7}$GpppA	$m_3^{2,2,7}$GMP + ADP	2.36 ± 0.16	2.06 ± 0.10	0.87 ± 0.07
HPLC method				
$m_2^{7,2'\text{-}O}$GpppG	$m_2^{7,2'\text{-}O}$GMP + GDP	39.77 ± 3.07	2.45 ± 0.11	0.06 ± 0.01
$m_2^{7,3'\text{-}O}$GpppG	$m_2^{7,3'\text{-}O}$GMP + GDP	13.87 ± 0.48	0.31 ± 0.10	0.02 ± 0.01
$m_3^{2,2,7}$GpppG	$m_3^{2,2,7}$GMP + GDP	3.91 ± 0.82	3.14 ± 0.32	0.80 ± 0.19

plasticity within the *C. elegans* DcpS cap-binding pocket.

We also examined $m_2^{7,2'\text{-}O}$GpppG and $m_2^{7,3'\text{-}O}$GpppG (bearing additional methylation at the 2′ or 3′ oxygen of m^7Guo) as *C. elegans* DcpS substrates (Fig. 3). K_m values determined by the fluorimetric and HPLC methods for both compounds are significantly higher than for m^7GpppG (Table 1). Furthermore, for $m_2^{7,3'\text{-}O}$GpppG, the rate of hydrolysis is drastically reduced. This compound has been studied previously as an effective inhibitor of $m_3^{2,2,7}$GpppA hydrolysis catalyzed by *C. elegans* DcpS, with K_i = 1 μM [26], significantly lower than the K_m value (∼ 14 μM) determined in this study (Table 1). Such a low K_i value indicates tight binding of $m_2^{7,3'\text{-}O}$GpppG with DcpS, whereas K_m involves a contribution from the dissociation step, including product release, which may be very slow in $m_2^{7,3'\text{-}O}$GpppG hydrolysis. As the inhibition type has not been determined, it is not obvious that $m_3^{2,2,7}$GpppA and $m_2^{7,3'\text{-}O}$GpppG compete for the same binding site in the inhibitory experiment [26].

The kinetic parameters obtained for $m_2^{7,2'\text{-}O}$GpppG and $m_2^{7,3'\text{-}O}$GpppG indicate that the 2′-OH and 3′-OH positions in the ribose ring of the m^7Guo moiety play a significant role in the catalytic activity of *C. elegans* DcpS.

Discussion

A series of modified dinucleotide cap analogs studied in this work defined several structural requirements for substrate specificity towards *C. elegans* DcpS. We found that cleavage of the cap structure occurs exclusively between β- and γ-phosphate groups in the triphosphate chain. We examined the ability of the enzyme to act on various cap analogs in a quantitative manner, employing two independent methods (fluorescence and HPLC) to determine the kinetic data.

Monomethylated and trimethylated natural substrates

Among the different scavengers investigated (human, nematode, yeast), *C. elegans* DcpS has a unique property, i.e. the possibility to hydrolyze both monomethylated (m^7GpppG and m^7GpppA) and trimethylated ($m_3^{2,2,7}$GpppG and $m_3^{2,2,7}$GpppA) cap structures. Our kinetic data demonstrate that trimethylated caps are cleaved with higher rates than their monomethylated counterparts (Table 1). However, MMG caps are recognized with higher specificity, indicating that the two additional methyl groups at the N2 position in TMG caps account for the differences in K_m for these substrates.

Substrates with an alkyl group at the N7 position

In agreement with previous data for nematode and human DcpS [14,20], we observed very low activity of *C. elegans* DcpS for the unmodified dinucleotide GpppG (Fig. 2). These results clearly show that, for tight and specific binding of the base moiety to the enzyme, the positive charge is required at the N7 position, introduced by a methyl or any alkyl group.

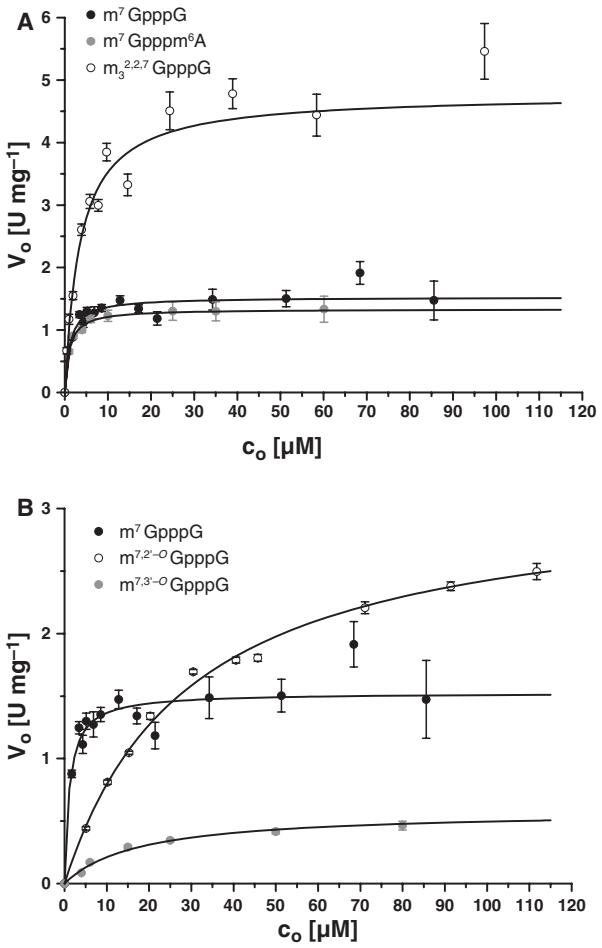


Fig. 3. *Caenorhabditis elegans* DcpS hydrolysis kinetics with cap analogs. (A) Comparison of the kinetic curves of *C. elegans* DcpS natural substrates (m^7GpppG, $m_3^{2,2,7}$GpppG) and a cap analog with a modification in the first transcribed nucleoside (m^7Gpppm^{6}A). (B) Comparison of the kinetic curves of m^7GpppG and anti-reverse cap analogs ($m_2^{7,2'-O}$GpppG and $m_2^{7,3'-O}$GpppG). The initial velocity data $v_o(c_o)$ were obtained from fluorescence studies.

Differently sized substituents (methyl, ethyl, benzyl) introduce positive charge into the base moiety, which is a key feature for the recognition of the cap structure. The amino acids involved in the stacking interactions with the methylated base are not conserved in different organisms (Fig. 4), and thus apparently are not crucial for hydrolytic activity, as indicated by the mutation L206A retaining over 90% of the wild-type activity of human DcpS [20]. The substrate properties of N7 alkylated dinucleotides (m^7GpppG, et^7GpppG, bn^7GpppG) do not differ significantly, as indicated by the kinetic parameters presented in Table 1. These data indicate that the cap-binding pocket of *C. elegans* DcpS is inherently flexible and able to accommodate different cap structures. This flexibility may explain why significantly large groups, such as ethyl or benzyl, can interact with nematode scavenger and be hydrolyzed with comparable rates.

Substrates modified in the first transcribed nucleoside

To investigate the catalytic mechanism of *C. elegans* DcpS with respect to the first transcribed nucleoside of the cap structure, we made a detailed quantitative comparison of the kinetic parameters for various cap analogs modified in the first transcribed nucleoside. We established that modifications introduced into the first transcribed nucleoside do not influence significantly nematode DcpS kinetic parameters. The substitution of adenine for guanine in m^7GpppG or $m_3^{2,2,7}$GpppG does not affect the K_m values. Other cap analogs bearing modifications of Guo, such as m^6A, m^7G, $m^{2'-O}$G and 2′dG, have similar kinetic parameters to m^7GpppG, indicating that modifications of the base or ribose moiety within the first transcribed nucleotide are not crucial for substrate recognition or the rate of hydrolysis. Moreover, the K_m value for m^7GpppG (1.17 ± 0.14 μM) is remarkably similar to the K_m value reported for m^7GpppBODIPY (1.21 ± 0.05 μM), containing an artificial fluorescent probe BODIPY instead of guanine [25]. *Caenorhabditis elegans* DcpS thus can accept different, even nonbiological substituents instead of the first transcribed nucleotide, which do not affect the substrate specificity or hydrolysis rate.

A similar effect was observed for human DcpS. Mutagenesis of the human DcpS amino acids responsible for the contacts with the first transcribed nucleoside had little effect on enzyme activity, suggesting that the structure of the binding pocket recognizing the first transcribed nucleoside is more flexible than that of the cap-binding pocket [20]. As shown in Fig. 4, the amino acids recognizing the first transcribed nucleoside are not conserved in DcpS homologs, indicating that interaction with this nucleoside is not very important for decapping activity. We thus propose that DcpS proteins exhibit structural plasticity for the first transcribed nucleoside, which has no affect on enzyme hydrolysis.

Substrates modified by additional methylation at the 2′ or 3′ oxygen of m^7Guo

The kinetic parameters obtained for $m_2^{7,2'-O}$GpppG and $m_2^{7,3'-O}$GpppG demonstrated the crucial role of the 2′-OH and 3′-OH groups of the m^7Guo moiety for *C. elegans* DcpS hydrolysis. The 2′-*O*-Me and 3′-*O*-Me

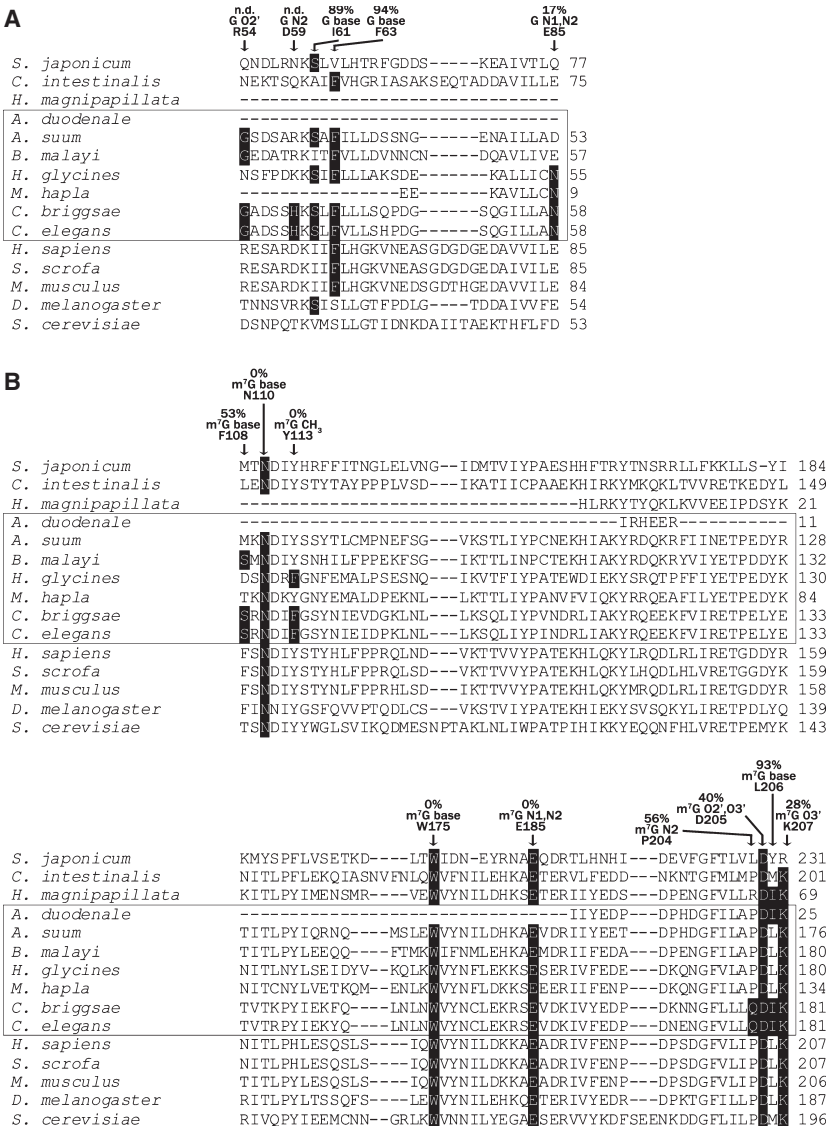


Fig. 4. Multiple sequence alignment of DcpS from different organisms generated using the CLUSTAL 2.0.12 program. The nematodes (*Ancylostoma duodenale*, *Ascaris suum*, *Brugia malayi*, *Heterodera glycines*, *Meloidogyne hapla*, *Caenorhabditis briggsae*, *Caenorhabditis elegans*) are framed. All the nematodes and the first three organisms (*Schistosoma japonicum*, *Ciona intestinalis*, *Hydra magnipapillata*) show trans-splicing, suggesting that they would probably be able to hydrolyze the TMG cap. The remaining orthologs are from *Homo sapiens*, *Sus scrofa*, *Mus musculus*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*. The amino acids of each organism are numbered on the right. Human DcpS (hDcpS) amino acids making vicinal or van der Waals' contacts with m^7GpppG are marked by arrows. The parts of m^7GpppG involved in these interactions and the percentage of m^7GpppG hydrolysis catalyzed by hDcpS with mutation of these amino acids to Ala are given above (n.d., not determined) [20]. Among the indicated amino acids, those identical to those in *C. elegans* DcpS are boxed in black. (A) Alignment of the amino acids involved in the interactions with the first transcribed nucleoside (Guo) in the hDcpS–m^7GpppG complex. These amino acids are not conserved in the other DcpS proteins illustrated. Mutation of the indicated amino acids in hDcpS to Ala only decreases slightly the enzymatic activity of the human scavenger [20]. (B) Alignment of the amino acids involved in the interactions with the cap structure (m^7Guo) in the hDcpS–m^7GpppG complex. The majority of these amino acids are highly conserved within the presented organisms. Mutations of these amino acids in human DcpS significantly or even completely inactivate the human enzyme [20].

analogs are so-called ARCA (anti-reverse cap analogs) which are commercially available and used as substrates for *in vitro* transcription reactions [27,28]. Such analogs prevent their reverse incorporation into mRNAs, thus producing transcripts which are more efficiently translated than those prepared with m^7GpppG. The transcripts obtained by this method are commonly used for numerous studies because they

mimic well natural transcripts, e.g. in the initiation of translation (the methylation of ribose of m^7Guo does not disturb the interaction with eIF4E) [28]. We established that, in some studies, ARCA-prepared transcripts may not be a good mimic of natural transcripts (this DcpS study is a good example). As indicated by the high K_m values and very low V_{max}/K_m values, both of these compounds are poor substrates for *C. elegans* DcpS. Interestingly, 2′-*O*- and 3′-*O*-methylations produce various susceptibilities of the cap to enzymatic hydrolysis. Despite the fact that the efficiencies of hydrolysis are reduced by two orders of magnitude compared with the natural substrates, the kinetic parameters (K_m and V_{max}) are significantly different. Although the leaving group is the same as in the MMG cap (GDP), the rate of hydrolysis observed for $m_2^{7,3'\text{-}O}$GpppG is significantly lower, suggesting that slow dissociation of the enzyme–product complex might be a controlling step in the hydrolysis process. With respect to substrate specificity, the loss of a hydrogen bond with the CH_3 substitution is more important in the 2′-*O*-position, leading to a significant reduction in substrate specificity. These results provide the first evidence indicating that 2′-*O*- and 3′-*O*-methylations of m^7Guo may influence the action of cap-binding proteins in a different manner. Our new finding could be a good starting point for the elucidation of the detailed mechanism of action on a molecular level, for the study of inhibition and for the design of effective inhibitors (in particular, human DcpS has been selected as a therapeutic target for spinal muscular atrophy treatment [29]). Moreover, the differences between the hydrolytic activities of $m_2^{7,2'\text{-}O}$GpppG and $m_2^{7,3'\text{-}O}$GpppG may be crucial for their biotechnological application.

The crucial role of the region associated with the binding of the ribose moiety also arises from a sequence alignment of different DcpS proteins (Fig. 4). The amino acids interacting with m^7Guo in human DcpS (Asn110, Trp175, Glu185, Asp205, Lys207) are highly conserved in the illustrated organisms. Mutations of these crucial amino acids resulted in enzyme inactivation or a significant decrease in activity [20]. Two amino acids, Asp205 and Lys207, are involved in interactions with the 2′-*O*- and 3′-*O*-positions of the ribose moiety of m^7Guo in the human protein.

Biological aspects

DcpS orthologs reported in different species (human, yeast and nematode cells) share significant sequence similarity (Fig. 4); however, they differ in their ability to hydrolyze different cap structures. Yeast and human scavengers recognize only monomethylated cap analogs as substrates, whereas *C. elegans* DcpS is capable of efficient cleavage of both MMG and TMG caps. Kinetic data for the enzymatic hydrolysis of m^7GpppG catalyzed by *S. cerevisiae* Dcs1 (K_m = 0.14 μM) [30] and *C. elegans* DcpS (K_m = 1.3 μM) (Table 1) illustrate their high specificity for the MMG cap. From such low K_m values, it can be concluded that DcpS enzymes are capable of maintaining high specific hydrolytic activity down to submicromolar intracellular concentrations of capped dinucleotides and short mRNA fragments. It therefore seems to be appropriately adapted to clear various capped species from the cells.

Despite their well-known decapping function in cytoplasmic mRNA turnover, yeast and human scavengers have been detected predominantly in the nucleus [13]. This may suggest that yeast and mammalian DcpSs are involved primarily in the nuclear degradation of the cap structure. Their high specificity for the MMG cap is crucial for the rapid removal of methylated nucleotides from the nucleus, preventing their misincorporation into the RNA chain during transcription [30]. In contrast, nematode DcpS is predominantly a cytoplasmic protein [15]. Although some regions of more intense DcpS labeling have been observed, DcpS scavengers are not components of specific degradation foci–processing bodies. The fact that *C. elegans* mRNAs are, in the majority (~70%), trimethylated may explain why most of the detectable DcpS protein is observed in the cytoplasm [15] and the higher hydrolytic activity towards the TMG cap determined in this study (Table 1). Dual activity of *C. elegans* DcpS is required for efficient degradation of mono- and trimethylated species, which may interact with eIF4E proteins during translation.

The ability of DcpS proteins to compete with eIF4E for the cap structure supports the idea that DcpSs may play modulatory roles at different levels of mRNA metabolism (cap-dependent translation, miRNA-guided translation repression, 5′ → 3′ degradation). Recently, it has been demonstrated that human DcpS is a nucleocytoplasmic shuttling protein with a broad functionality as a modulator of cap-dependent processes [30]. It has also been suggested that decapping activity in *C. elegans* and *S. cerevisiae* is required for responses to heat shock and genotoxic stress [25,31].

The kinetic studies presented in this article provide insight into the mechanism of interaction of MMG and TMG caps with the binding pocket of *C. elegans* DcpS. The detailed characteristics of the DcpS scavenger presented in this study are essential to understand the key step in mRNA turnover, and may enable the design and synthesis of new cap analogs that are

selective inhibitors for parasitic nematode DcpSs, without affecting their mammalian counterparts.

Materials and methods

Materials

Recombinant *C. elegans* DcpS in pET16b [14] was grown in *Escherichia coli* Rosetta (DE3) cells (Novagen, Madison, WI, USA) at 37 °C until an absorbance at 600 nm (A_{600}) of 0.5 was reached. Protein production was induced by the addition of 0.4 mM isopropyl thio-β-D-galactoside (IPTG) and by shaking the bacterial culture for 16 h at 20 °C. The culture was centrifuged and the bacterial pellets were resuspended in ice-cold lysis buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 300 mM urea, 10% glycerol, 1% Triton X-100, 10 mM imidazole); lysozyme was added to a final concentration of 1 mg·mL^{-1}, the suspension was incubated on ice for 30 min, and then sonicated on ice (15 × 30 s every 1 min). The 6 × His-tagged DcpS was bound to Ni^{2+} - nitrilotriacetic acid (NTA)-agarose (Novagen) for 60 min at 4 °C, and unbound proteins were removed with washing buffer (20 mM Tris/HCl, pH 7.5, 300 mM NaCl). The bound protein was eluted with 2 mL portions of elution buffer (20 mM Tris/HCl, pH 7.5, 300 mM NaCl) containing increasing concentrations of imidazole (20–300 mM). Fractions containing DcpS activity were dialyzed against 20 mM Tris/HCl, pH 7.6, 50 mM KCl, 0.2 mM EDTA, 20% glycerol and 1 mM dithiothreitol, and stored at –80 °C. The enzyme activity was checked before each set of experiments. The concentration of DcpS was estimated by the method of Bradford [32] and spectrophotometrically from its molar absorption coefficient ε_{280} = 38 900 M^{-1}·cm^{-1} (calculated from the amino acid composition of a monomer using an algorithm on the ExPASy Server).

The cap analogs investigated in this work (m^7GpppG, $m_3^{2,2,7}$GpppG, m^7GpppA, $m_3^{2,2,7}$GpppA, $m_2^{7,2'\text{-}O}$GpppG, $m_2^{7,3'\text{-}O}$GpppG, bn^7GpppG, et^7GpppG, m^7Gpppm^{7}G, m^7Gppp2′dG, m^7Gpppm$^{2'\text{-}O}$G, m^7Gpppm^{6}A) were prepared according to the methods described earlier [27,28,33–36].

Analysis of hydrolysis kinetics

Dinucleotide cap analogs and their hydrolysis products were identified using absorption and emission spectroscopy and HPLC analysis. The concentrations of the investigated substrates were determined on the basis of their absorption coefficients: $\varepsilon_{255}(m^7\text{GpppG})$ = 22 600 M^{-1}·cm^{-1}; $\varepsilon_{259}(m^7\text{GpppA})$ = 21 300 M^{-1}·cm^{-1}; $\varepsilon_{262}(m^7\text{Gpppm}^6\text{A})$ = 21 100 M^{-1}·cm^{-1}; $\varepsilon_{259}(m^7\text{Gpppm}^7\text{G})$ = 16 000 M^{-1}·cm^{-1}; $\varepsilon_{255}(m^7\text{Gpppm}^{2'\text{-}O}\text{G})$ = 19 600 M^{-1}·cm^{-1}; $\varepsilon_{255}(m^7\text{Gppp2}'\text{dG})$ = 19 300 M^{-1}·cm^{-1} [37]; $\varepsilon_{255}(m_2^{7,2'\text{-}O}\text{GpppG})$ = 20 800 M^{-1}·cm^{-1}; $\varepsilon_{255}(m_2^{7,3'\text{-}O}\text{GpppG})$ = 22 000 M^{-1}·cm^{-1} (J. Zuberek, Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Poland, unpublished data); $\varepsilon_{255}(et^7\text{GpppG})$ = 21 900 M^{-1}·cm^{-1}; $\varepsilon_{256}(bn^7\text{GpppG})$ = 17 800 M^{-1}·cm^{-1}; $\varepsilon_{258}(m_3^{2,2,7}\text{GpppG})$ = 26 300 M^{-1}·cm^{-1} [36]. The coefficient for $m_3^{2,2,7}$GpppA (ε_{260} = 28 900 M^{-1}·cm^{-1}) was calculated in this study. Absorption spectra were recorded in 0.1 M phosphate buffer, pH 7.0, on a Lambda 20UV/VIS spectrophotometer (Perkin-Elmer, Waltham, MA, USA) at 20 °C.

The hydrolytic activity of the recombinant *C. elegans* DcpS was assayed at 20 °C in 50 mM Tris buffer containing 20 mM $MgCl_2$ and 30 mM $(NH_4)_2SO_4$ (final pH 7.2). DcpSs have been reported to share a neutral pH range (pH 7–8) as the optimum reaction medium for their activity [14,25,26]. We have demonstrated previously that the kinetic parameters of enzymatic hydrolysis catalyzed by *C. elegans* DcpS do not change significantly in this pH range [26]. However, the fluorescence intensity and stacking interactions of dinucleotide cap analogs are strongly dependent on pH. The cationic (N1 protonated) form of the 7-alkylated residue exhibits a higher fluorescence quantum yield and more efficient stacking than its zwitterionic counterpart [38–40]. A lower pH is thus more favorable for the observation of the fluorescence increase during the cleavage of the pyrophosphate bridge. Consequently, pH 7.2 was adopted for the enzymatic hydrolysis assays monitored by the fluorimetric method, as well as for the HPLC measurements.

The initial substrate concentration ranged from 0.5 to 120 μM, depending on the analyzed compound. DcpS cleavage assays were carried out with 0.11–1.98 μg of the recombinant protein. The products of enzymatic hydrolysis were examined by analytical HPLC (Agilent Technologies 1200 Series, Santa Clara, CA, USA) using a reverse-phase Supelcosil LC-18-T column (4.6 mm × 250 mm, 5 μm) and a UV/VIS and fluorescence detector. After sample injection, the column was eluted at room temperature with a linear gradient of methanol from 0% to 25% in aqueous 0.1 M KH_2PO_4 over 15 min at a flow rate of 1.3 mL·min^{-1}. The fluorescence at 337 nm (excitation at 280 nm) and absorbance at 260 nm were continuously monitored during the analysis.

For all investigated dinucleotides, the spectrofluorimetric method was used to determine the kinetic parameters. The fluorescence measurements were performed on an LS 55 spectrofluorometer (Perkin-Elmer) in a quartz cuvette (Hellma, Müllheim, Germany) with an optical path length of 4 mm for absorption and 10 mm for emission. The fluorescence intensity was observed at 380 nm (excitation at 294–318 nm, depending on the cap analog) and corrected for the inner filter effect. Hydrolysis was followed over 10 min by recording the time-dependent increase in fluorescence intensity caused by the removal of intramolecular stacking as a result of enzymatic cleavage of the triphosphate bridge. The substrate concentration (c) at the time of hydrolysis (t) was calculated as:

$$c = c_o(I_t - I_e)/(I_o - I_e)$$

where c_o is the initial concentration of the substrate, and I_t, I_o and I_e are the fluorescence intensities at time t, at the beginning and at the end of the reaction, respectively. The initial velocity (v_o) of each reaction was calculated by the linear regression of the substrate concentration versus time.

In order to confirm the fluorimetric data, the kinetic parameters for $m_3^{2,2,7}$GpppG, $m_2^{7,2'-O}$GpppG and $m_2^{7,3'-O}$GpppG were also obtained by HPLC. Other cap analogs could not be studied using chromatographic analysis, because the sensitivity of the HPLC system was not adequate to detect the very low substrate concentrations (0.2–10 μM) necessary to determine K_m values of ~ 1 μM. HPLC analysis is more effective for kinetic studies of compounds characterized by higher K_m values (> 10 μM). In the HPLC procedure, buffer solutions containing the respective dinucleotides were incubated at 20 °C for 10 min. The hydrolysis process was started by the addition of DcpS. At 3 or 5 min time intervals, 150 μL aliquots of the reaction mixture were withdrawn and the reaction was terminated by heat inactivation of the enzyme (2.5 min at 100 °C). The samples were then subjected to HPLC analysis as described above. The concentration of the examined compounds during the course of hydrolysis was determined from the area under the chromatographic peaks, using the following formula:

$$c = c_o(1 - x)$$

where c is the substrate concentration at the time of hydrolysis (t), c_o is the initial substrate concentration and x is the extent of decapping measured as the percentage of hydrolyzed substrate.

The initial velocity method was used to calculate the kinetic parameters for both the fluorimetric and HPLC methods. The initial velocity (v_o) of each reaction was calculated by the linear regression of the substrate concentration versus time. The K_m and V_{max} values were determined from hyperbolic fits to the Michaelis–Menten equation by nonlinear regression using ORIGINPRO 7.0 (Microcal Software, Northampton, MA, USA).

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