MECHANISTIC AND KINETIC ANALYSIS OF THE DCPS SCAVENGER DECAPPING ENZYME

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Decapping is an important process in the control of eukaryotic mRNA degradation. The scavenger decapping enzyme DcpS, functions to clear the cell of cap structure following decay of the RNA body by catalyzing the hydrolysis of m\(^7\)GpppN to m\(^7\)Gp and ppN. Structural analysis has revealed that DcpS is a dimeric protein with a domain swapped amino terminus. The protein dimer contains two cap binding/hydrolysis sites and displays a symmetric structure with both binding sites in the open conformation in the ligand-free state, and an asymmetric conformation with one site open and one site closed in the ligand-bound state. The structural data are suggestive of a dynamic decapping mechanism where each monomer could alternate between an open and closed state. Using transient state kinetic studies, we show that both the rate limiting step and rate of decapping are regulated by cap substrate. A regulatory mechanism is established by the intrinsic domain swapped structure of the DcpS dimer such that the decapping reaction is very efficient at low cap substrate concentrations yet, regulated with excess cap substrate. These data provide biochemical evidence to experimentally verify a dynamic and mutually exclusive cap hydrolysis activity of the two cap binding sites of DcpS and provide key insights into its regulation.

INTRODUCTION

The control of mRNA degradation is a critical step in the posttranscriptional regulation of gene expression, and the steady state level of any mRNA species depends on both the rate of mRNA synthesis and its breakdown. In eukaryotic cells, cytoplasmic mRNA degradation proceeds predominantly through an initial removal of the polyadenylated tail (1,2) followed by 5' to 3' or 3' to 5' decay (3,4). In the 5'-3' decay pathway, the 5' cap structure is cleaved by the catalytic activity of the Dep2 decapping enzyme to release the m\(^7\)GDP and monophosphorylated RNA (5-8). The resulting uncapped monophosphorylated RNA is digested by a 5'-3' exonuclease, Xrn1 (1,9). In the 3'-5' pathway, subsequent to deadenylation the capped-RNA body is continuously degraded by an exosome complex (10,11). The resulting capped oligonucleotide m\(^7\)GpppN(pN)_n (n<9) is hydrolyzed by a second type of decapping enzyme, DcpS, to release the m\(^7\)Gp and ppN(pN)_n products (12,13). These pathways need not be mutually exclusive and could occur simultaneously (14,15) and an interplay between the two pathways could also exist. DcpS, which was originally characterized as the decapping enzyme in the 3'-5' pathway, is also able to hydrolyze the 5'-3' decapping product m\(^7\)GDP to release m\(^7\)Gp (16,17). Furthermore, disruption of yeast DcpS ortholog, Dcs1p, impeded the 5' exonuclease activity (18), indicating the Dcs1p decapping products might serve as signaling molecules for the 5' decay pathway.

DcpS is a member of the Histidine triad (HIT) hydrolase family of proteins that contain a stretch of His-X-His-X-His-X residues, where X denotes hydrophobic amino acid residues. HIT proteins are dimeric nucleotide binding proteins that have hydrolase activities (19-21). The central histidine residue is critical for the hydrolase activity as it is thought to serve as the nucleophile attacking the phosphate most proximal to the methylated guanosine in m\(^7\)GpppG (22) and is also critical for DcpS hydrolisis (13).

Structural analysis of DcpS has revealed that it is a homodimer with a symmetric structure when in the ligand free form (16,23), or asymmetric...
homodimer in the ligand bound form (16,24). Each DcpS monomer possesses a distinct N terminal domain and a C terminal domain containing the HIT motif linked by a hinge region. The N-terminal domain displays a domain swapped conformation by exchanging an identical α-helix and two anti-parallel β-strands with the second monomer. The DcpS homodimer contains two cap binding pockets which serve as the active sites for cap hydrolysis. In the ligand bound form, DcpS forms a closed conformation on one side and an open conformation on the other, with substrate bound at the C-terminal domain of each side (24). The structure suggests that the closed conformation constitutes the cap hydrolysis productive site while the open site would be nonproductive. The N terminal domains can either both be in the open state or one side in an open state with the second in a productive closed state with the hinge enabling the N-terminus to flip back and forth, alternating on each side (16,24).

Here, we examined the enzyme kinetics of DcpS and demonstrate substrate binding and hydrolysis are regulated by negative cooperativity. Furthermore, by comparing the enzymatic behaviors of the wild type homodimer of DcpS and HIT mutant heterodimer, we biochemically confirm and expand upon the previously proposed dynamic mechanism of decapping implied by the structural analysis (16,24).

EXPERIMENTAL PROCEDURES

Plasmid constructs

The pcDNA3-Flag-DcpS plasmid, which expresses Flag-tagged DcpS in HEK293T cells, was constructed by inserting the DcpS open reading frame flanked by a *BamH* I site at the 5′ end and an *Xho* I site at the 3′ end into the pcDNA3-Flag vector (25). A second Flag-tagged DcpS expression plasmid, pcDNA3-FlagDcpS-2, was constructed by inserting the DcpS open reading frame (ORF) with a Flag tag fragment at the 5′ of the ORF into the *Neo* *Xho* I sites of pcDNA3 vector (Invitrogen; Carlsbad, CA). Plasmids expressing a series of double-tagged homodimer and heterodimer proteins were generated by inserting two corresponding tagged wild-type or mutated DcpS ORFs into the two multiple cloning regions of the commercial plasmid pETDuet-1 vector (Novagen; San Diego, CA). Plasmid pETDuet-1HisDcpSFlagDcpS was constructed by inserting the *Nco* I (Klenow filled)/*Xho* I restriction fragment from pcDNA3-FlagDcpS-2, as well as the *Neo* *I/Xho* I(13) restriction fragment from pET28-DcpS into the *EcoR* *V/Xho* I sites and *Neo* *I/EcoR* I (Klenow filled) sites in pET-Duet-1, respectively. Plasmids pETDuet1-HisDcpS*H277N*FlagDcpS and pETDuet1-HisDcpS*W175A*FlagDcpS*N110A* were constructed in a similar way, except with the indicated mutated sites introduced into the DcpS ORF. The mutagenesis of DcpS H277N was described in (13). The mutagenesis of N110A and W175A was carried out using the QuickChange mutagenesis system (Stratagene; La Jolla, CA) with the primer sets 5′-CTC CAA TTG CAG TTC TCC GCT GAT ATA TAT CAG CGG AGA ACT GCA ATT GGA G-3′, 5′-GAT AGG TGC TGT ATA TAT CAG CGG AGA ACT GCA ATT GGA G-3′, and 5′-CAG ACG CTC AGC ATC CAG GCG GTG TAT AAC ATT CTC GAC-3′, 5′-GTC GAG AAT GTT ATA CAC CGC CTG GAT GAG GCT GAT GCT CTG G-3′, respectively.

The single tagged homodimer proteins His-DcpS*BC/BC* and Flag-DcpS*BC/BC* were used in Figure 4C and contain the N110A and W175A double mutations. They were expressed from plasmids pET28-DcpS*N110AW175A* and pcDNA3 Flag DcpS*N110AW175A*, respectively. These two plasmids were generated by introducing the N110A and W175A mutations into the plasmids pET28-DcpS and pcDNA3-FlagDcpS-2 respectively, using the primer sets described above.

Recombinant protein expression and purification

Recombinant Flag DcpS was purified from HEK293T cells transfected with pcDNA3-Flag-DcpS by using Anti-Flag M2 agarose beads (Sigma, St. Louis, MO). 1 x 10^8 293T cells were transfected with 120 µg pcDNA3-flag DcpS and incubated at 37°C, 5% CO_2 to allow transient protein overexpression. Forty-eight hours posttransfection, cells were harvested and washed with 1 x PBS and resuspended in sonication buffer (150 mM KCl, 20 mM Tris HCl pH 7.9, 0.2 mM EDTA, 10% glycerol, 0.01% NP40) followed by sonication for 30 seconds. The cell debris were then spun down, and the supernatant was incubated with 200 µl Anti-Flag M2 agarose beads at 4°C for 3 hours with mild shaking to allow the Flag-DcpS protein bind to the beads. The beads contamination.
were then washed twice with 10 ml wash buffer (300 mM KCl, 20 mM Tris HCl pH 7.9, 0.2 mM EDTA, 10% glycerol, 0.025% NP40). The bound Flag-DcpS proteins were eluted by 1 ml elution buffer (wash buffer with 100 µg/ml Flag peptide, Sigma; St. Louis, MO). The eluted proteins were then concentrated by Centricon centrifugal filter columns (Amicon; Bedford, MA).

The expression of double tagged recombinant homodimer and heterodimer proteins was performed using the pETDuet-1 expression system. Plasmids pETDuet1-HisDcpS/FlagDcpS, pETDuet1-HisDcpS1277N/Flag-DcpS, and pETDuet1-HisDcpS1175A/FlagDcpS1110A were used to transform BL21-CodonPlus(DE3)-RIPL competent cells (Stratagene; La Jolla, CA) according to the manufacturer’s instructions to generate the DcpS\(^{WT/WT}\) DcpS\(^{WT/HT}\) and DcpS\(^{WT/BC}\) proteins respectively. A single colony was picked and grown in two liters LB medium containing 100 mg/ml ampicillin and 20 mg/ml chloramphenicol to 0.6 OD\(_{600}\) and induced with 0.2 mM IPTG at 30°C for 2-3 hours. The bacterial cells were then washed and resuspended in binding buffer (300 mM urea, 5mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) and sonicated for three times for 30 sec at 1 min intervals in ice. The cell debris was then spun down and the cell lysate containing the recombinant homo- or heterodimer proteins were subjected to a two-step affinity purification procedure. The recombinant proteins were first purified on a nickel charged column for His tagged protein binding, according to the manufacturer except there was 300 mM urea and 0.5% Triton X-100 included in the binding buffer. The bound His-tagged proteins were eluted in 1.5 ml elution buffer (500 mM imidazole, 500mM NaCl, 20 mM Tris-HCl pH 7.9) from the nickel column. The eluted proteins were then subjected to the second affinity column. The eluate was slowly added into 15 ml Flag IP binding buffer (150 mM KCl, 20 mM Tris HCl pH 7.9, 0.2 mM EDTA, 10% glycerol, 0.01% NP40) and incubated with 200 µl prewashed Anti-Flag M2 agarose beads at 4°C for 3 hours with mild shaking. The bound proteins were then washed with wash buffer and eluted as for Flag DcpS proteins. The eluted proteins are His and Flag double tagged homo- or heterodimers since they underwent both His and Flag column purification. They were subsequently concentrated by Centricon centrifugal filter columns (Amicon; Bedford, MA).

Recombinant protein concentrations were determined by spectrophotometry and calculated based on their extinction coefficient (26) obtained from th ExPASY ProtParam webtool (27). Due to the relative purity of the protein sample shown in Fig 1A the detected absorbance was directly used with the DcpS extinction coefficient to determine the concentration. Protein samples shown in Fig 3B and 4B contained a copurified bacterial protein, GroEL. Therefore, extinction coefficients of both proteins were used in the calculations and the corresponding concentrations of the DcpS dimers were determined.

**Generation of cap structures**

Unlabeled, uncapped RNA corresponding to the pcDNA3 polylinker spanning from the SP6 promoter to the T7 promoter (pep) with 16 guanosines at the 3’ end was transcribed by SP6 RNA polymerase from a PCR-generated template using the primers 5’-CGATTAGGTGACACTATAG-3’ and 5’-CCCCCCCCCCCCCCCCCAGACTCACTATAG-3’. Cap labeled RNA was generated with the vaccinia virus capping enzyme utilizing \(\alpha-^{32}P\) GTP and S-adenosyl-methionine (SAM) to label the first phosphate within the cap relative to the methylated guanosine (m7G*pppG-) and the RNA gel-purified as described (28). Labeled cap structure without the RNA body was generated by treating the cap-labeled RNA with 1 unit Nuclease P1 (Sigma; St. Louis, MO) for 1.5 h at 37°C to hydrolyze the RNA body leaving the intact cap structure as described (12).

**In vitro decapping assays and data analysis**

Decapping assays in Figure 4C were carried out with the indicated monomer concentrations of proteins and 200 nM cold cap structure (New England Biolab; Beverly, MA) spiked with \(^{32}P\)-labeled cap structure in decapping buffer (10 mM Tris pH 7.5, 100 mM KOAc, 2 mM MgOAc, 2 mM DTT) for 30 sec at room temperature. Decapping reactions were terminated by 1.7 N formic acid. An aliquot of each reaction was spotted onto PEI-cellulose TLC plates (Sigma; St. Louis, MO) that were prerun in H\(_2\)O and air dried, and the products were developed with 0.45 M (NH\(_4\))\(_2\)SO\(_4\) at room temperature. The TLC plates
were air dried and exposed to PhosphorImager for quantitation. All quantitations were conducted with a Molecular Dynamics PhosphorImager (Storm860) using ImageQuant-5 software.

The decapping assays were conducted on a rapid quench-flow instrument (KinTek Corp., Austin, TX) at 25°C. DcpS was loaded in one syringe of the quench-flow apparatus. The cold cap substrate spiked with radiolabeled cap substrate with defined concentrations as indicated, were loaded in a second syringe. Decapping reactions were initiated by rapidly mixing equal volumes of solutions from both syringes. After the mixtures of enzyme and substrate were incubated for the indicated times, the reactions were quenched by 2.3 N formic acid added from a third syringe. An aliquot of each reaction was spotted onto TLC plates, developed, dried and exposed to PhosphorImager and quantitated as above.

The kinetics in Figure 1C, 2A and 4D were fit to Equation 1 using SigmaPlot 10.0 software (Systat Software, Inc.; Point Richmond, CA)

\[ Y = Y_0 + A(1 - \exp(-k_{\text{obs}}t)) \]  
\[ \text{Eq 1} \]

\( Y \) is the fraction of product \( m^7 \text{Gp} \) over total substrate; \( Y_0 \) is the interception representing the fraction of background \( m^7 \text{Gp} \) hydrolyzed from the input substrate in the absence of enzyme; \( A \) is the fraction of product generated from actual cleavage of enzyme; \( k_{\text{obs}} \) is the observed rate constant of cap substrate hydrolysis, and \( t \) represents the reaction time.

The kinetics of DcpS\(^{\text{WT/HIT}}\) under excess enzyme over substrate (single turnover conditions) in Figure 3D were fit to Equation 2.

\[ Y = Y_0 + A(1 - \exp(-k_{\text{obs}}t)) + B(1 - \exp(-Ct)) \]  
\[ \text{Eq 2} \]

\( Y, Y_0, A, \) and \( k_{\text{obs}} \) represent the same parameters as that of Equation 1. \( B \) is the amplitude of the second phase in the kinetics, \( C \) is the rate constant of the second phase, and \( t \) is time.

**Electrophoretic mobility shift assay (EMSA)**

EMSA were carried out by incubating proteins with labeled cap structure in RNA binding buffer (RBB; 75 mM KCl, 10 mM Tris HCl, pH 7.5, 1.6 mM MgCl\(_2\), and 0.5 mM DTT) per reaction on ice for 15 min. The resulting protein-cap complexes were resolved on a 5.6% native polyacrylamide gel. The gel was dried and exposed to PhosphorImager.

**RESULTS**

**Hydrolysis of cap structure by DcpS is rate limiting at the binding step under single turnover conditions**

To gain a better understanding of the mechanism by which the DcpS scavenger decapping enzyme functions to hydrolyze cap structure, we undertook an enzymatic kinetic analysis of DcpS decapping. To measure the rate of the decapping reaction, \textit{in vitro} decapping assays were performed under conditions where enzyme concentration was in excess of the substrate concentration (single turnover conditions) and time courses were measured using a rapid chemical quench-flow apparatus. The DcpS monomer concentration was varied from 20 nM, 50 nM, 100 nM, and 200 nM and the cap substrate was kept constant at 10 nM and was spiked with \(\alpha^{32}\)P labeled cap to follow the decapping products and reactants. The label is exclusively at the first phosphate following the methylated guanosine to enable detection of both the cap structure substrate (m\(^7\text{Gp}^*\text{ppG}\)) and the decapping product (m\(^7\text{Gp}\*)

A Sypro Ruby (Invitrogen; Carlsbad, CA) stained gel of Flag-tagged DcpS purified from HEK293T cells is shown in Figure 1A. The kinetics of cap hydrolysis was monitored from 0.1 s to 2 min at 25°C. The substrate and product was resolved by polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) (Figure 1B) and the fraction of generated product was plotted against time (Figure 1C). The kinetics were fit to a single exponential equation (Equation 1, Experimental Procedures) that provided the decapping rate constants at each enzyme concentration (Table 1).

As shown in Figure 1D and summarized in Table 1, the observed decapping rate constants depended on the DcpS concentrations and increased linearly with increasing DcpS. This dependency indicates that the observed rate of decapping is limited by the formation of the initial enzyme-substrate complex. That is, the binding step is rate limiting under the conditions used where the enzyme is in excess of substrate. If the
rate limiting step occurred after the enzyme-substrate binding step, increasing the amount of enzyme would have no effect on the observed reaction rate. Thus, the data are consistent with the rate limiting step being the initial substrate binding step under the conditions of the experiments. We infer from these data that the decapping reaction rate constant is >1.3 s⁻¹ (Table 1). The bimolecular rate constant of substrate binding was obtained from the slope of the line in Figure 1D and is equal to 6.8 ±0.6 x10⁶ M⁻¹s⁻¹. To obtain the apparent dissociation constant (Kₐ), the amount of m⁷Gp product produced after the reaction reached equilibrium was plotted against the respective DcpS concentrations (20-200 nM). The saturated amplitudes indicate that the DcpS-cap complex K_d is <20nM (Figure 1E) consistent with the K_d of 75nM we previously reported (29).

The open to closed conformational step is the rate limiting step under high substrate conditions

The above kinetic analysis demonstrates that the binding between DcpS and cap substrate is the rate-limiting step at the concentrations used in the above experiments and where the enzyme is in excess over the substrate. We next tested parameters with excess substrate and limiting enzyme concentrations. The decapping reaction was performed in the rapid chemical quench-flow apparatus as above, except the DcpS monomer concentration of 100 nM and excess cap substrate concentrations of 200 nM, 400 nM, 800 nM and 1600 nM were used. The reaction products were resolved by thin-layer chromatography as in Figure 1B and plotted in Figure 2A. The kinetics of the decapping reaction under these multiple turnover reaction conditions was initially linear and then as substrate was depleted the kinetics became nonlinear. The kinetic curves in Figure 2B were fit to obtain the initial rate of decapping (Table 1). The initial rate (nM/s) was divided by [DcpS] to obtain the pseudo first order rate constants of decapping, which were plotted against the substrate concentrations (200-1600 nM). As shown in Figure 2B and Table 1, the increasing amount of cap substrate had no effect on the observed rate constants, which remained constant around 0.1 s⁻¹. This independency of rate on substrate concentration indicates that substrate binding is not rate limiting. Curiously, this observed rate of decapping under excess substrate conditions is about 109 times lower than what we expect from the rate measurements at 1.6 µM cap substrate under single turnover kinetic conditions. From the measured bimolecular rate constant of substrate binding equal to 6.8 ± 0.6 x10⁶ M⁻¹s⁻¹ and for 1.6 µM cap substrate concentration, we expect the observed rate constant close to 6.8 ± 0.6 x10⁶ M⁻¹s⁻¹ x 1.6 µM = 10.9 s⁻¹, which is 109 times higher than what we observed (0.1s⁻¹). The results indicate that there is a change in the rate limiting step and the decapping reaction is slower when the cap substrate concentration is higher than the DcpS concentration.

To identify the rate limiting step under conditions of excess cap over DcpS, we examined the presteady state portion of the kinetics of decapping in Figure 2A. When product release of a reaction is rate limiting, the reaction proceeds through an initial burst (with an amplitude close to the enzyme concentration) for the first round of substrate turnover. An initial steep slope with a subsequent shallow slope would indicate the reaction proceeds through an initial rapid burst, but the subsequent rounds of hydrolysis are slower due to a slow release of the product. The amplitude of the initial burst would be close to the active enzyme concentration (which we assume is close to 100 nM, the concentration of DcpS used in these reactions). The observed kinetics at all cap substrate concentration however fit to a single exponential curve with no obvious burst with an amplitude close to DcpS concentration. The kinetics therefore indicate that the product release is not a rate limiting slow step. Since substrate binding is also not rate-limiting, by process of elimination, a step between substrate binding and product release is the rate limiting step under excess substrate conditions. We propose that under excess substrate concentration when both sites of the DcpS dimer are occupied by substrate, the conformational change bringing one of the sites to the closed catalytically competent state is rate limiting.

A requirement for pivoting of the N-terminus for efficient cap binding and hydrolysis

Structural analysis of DcpS has revealed that it is a dimeric protein, with two active sites that are created with contributions from both the N-terminal and C-terminal domains (24). By virtue
of the hinge that separates the two domains, the N-terminus has the capacity to flip from one side to the other creating a closed complex bound to a cap substrate on one side to initiate hydrolysis which in turn would force an open conformation on the other side, and vice versa (24). Therefore, the structural data is consistent with a mutually exclusive activity of the two sites. If this proposed mechanism is true, both sides of the DcpS protein are not able to close at the same time. A prediction would be that when one side is locked into the closed conformation, the other side would remain open and would not be able to close freely to cleave its bound cap. To test this hypothesis biochemically, a heterodimer with one wild type active site and the other containing an asparagine substitution at His 277 in the HIT motif (DcpS\(^{WT/HIT}\)) which renders the protein inactive (13) was generated (Figure 3A, 3B).

To confirm the HIT mutant protomer within the DcpS\(^{WT/HIT}\) heterodimer has the capacity to bind cap structure, we tested its binding by an electrophoretic mobility shift assay. As shown in Figure 3C, lane 3, the DcpS\(^{WT/HIT}\) heterodimer was capable of binding cap structure. This binding is likely mediated through the HIT mutant protomer since a wild type protomer is expected to hydrolyze the cap and not stably bind it in this assay system. Consistent with this hypothesis, the wild type DcpS homodimer does not stably bind cap structure (lane 2) while as expected the DcpS H277N (DcpS\(^{HIT/HIT}\)) homodimer which can stably bind but not hydrolyze two cap structure molecules per homodimer protein (24) formed a detectable complex (lane 4). Therefore, the HIT mutant cap binding site within the DcpS\(^{WT/HIT}\) should contain the capacity to bind and trap the cap substrate without hydrolyzing it. We expect the HIT protomer to bind and possibly interfere with hydrolysis on the wild type side of the heterodimer.

Rapid quench-flow decapping assays were carried out with the DcpS\(^{WT/HIT}\) heterodimer under limiting substrate single turnover (100 nM protein monomer, 10 nM substrate) and excess substrate multiple turnover (100 nM protein monomer, 800 nM substrate) conditions and the decapping products were resolved by TLC. The data points from the single turnover experiments were fit to a biphasic, double exponential curve (Figure 3D), and the kinetic values were calculated from Equation 2 equation and listed in Table 2. Under single turnover conditions the DcpS\(^{WT/HIT}\) protein was catalytically active. The decapping amplitude reached 20% in a single exponential phase, compared to ~100% for the DcpS\(^{WT/WT}\) (compare Figure 3D and Figure 1C). These data suggest that 80% of the cap substrate was captured at the inactive HIT side, while only 20% was bound by the WT side and hydrolyzed. Moreover, the data suggest the HIT mutant side had a stronger affinity for the cap substrate and was able to bind and trap a majority of the cap substrate. The second exponential phase might represent the fact that substrates bound at the HIT side were slowly released and eventually hydrolyzed by the wild type side.

In contrast to the extent of decapping observed with limiting substrate, under conditions of excess substrate over enzyme, less then 2% of decapping was observed (Figure 3D). These latter data suggest that the HIT mutant side of almost all the DcpS dimers were bound and locked by the cap substrates, therefore the WT side was forced to remain in the open confirmation and unable to hydrolyze other cap substrates. These data confirm the proposed mechanism that the two N-termini of DcpS dimer cannot simultaneously hydrolyze the caps at both binding sites with both sites in the closed conformation, indicating that when one side is closed, the other side is forced open.

**Decapping by a DcpS heterodimer containing one binding compromised mutant subunit displayed decreased negative cooperativity**

To further confirm the negative cooperativity between the two active sites within the DcpS dimer, a DcpS heterodimer containing one wild-type active site and a mutated second site with minimal cap binding capacity and unable to hydrolyze cap substrate was generated. With a heterodimer containing only one active site and a second that was rendered binding compromised, we would expect there to be minimal cooperativity within this heterodimer since only one side of the protein can effectively interact with the cap substrate. To generate the heterodimer with one binding compromised site, mutants were constructed such that two residues within one active site of the dimer critical for cap structure binding were substituted with alanine to abolish
Decapping of the double tagged DcpS<sup>WT/WT</sup>, as well as the heterodimer with one wild type monomer and a second binding compromised monomer (DcpS<sup>WT/BC</sup>) was next tested. Decapping assays were carried out with DcpS<sup>WT/WT</sup> or DcpS<sup>WT/BC</sup> under single turnover conditions (100nM monomer concentration of DcpS enzyme, 10nM cap substrate) and multiple turnover conditions (100nM monomer concentration enzyme, 800nM cap substrate). The decapping products were resolved by TLC and the fraction of product generated determined and plotted in Figure 4D for the DcpS<sup>WT/BC</sup> heterodimer. The curves were fit to a single exponential equation (Equation 1). The rate constants and maximal fraction of generated decapping product were determined from the equation coefficients and listed in Table 2. We show that the rate constant for DcpS<sup>WT/WT</sup> under multiple turnover conditions, was reduced by 8 fold compared to the single turnover conditions (0.735 ±0.037 s<sup>-1</sup> vs. 0.0955 ±0.0009 s<sup>-1</sup>), suggesting the existence of negative cooperativity in the wild-type homodimer. Interestingly, under the same conditions, the rate constant of the heterodimer DcpS<sup>WT/BC</sup> was reduced by only 2 fold compared to the single turnover conditions (0.08 ±0.002s<sup>-1</sup> vs. 0.042 ±0.0009 s<sup>-1</sup>), indicating that higher concentrations of substrate could still negatively impact hydrolysis at the active site but the impact is significantly lower compared to that observed with the wild type homodimer. The failure of the DcpS<sup>WT/BC</sup> heterodimer to be completely resistant to negative cooperativity could be due to the ability of the double mutant site still retaining residual cap binding capacity (see Discussion).

**DISCUSSION**

In this report, we present kinetic analysis of DcpS and elucidate several novel insights into its decapping mechanism at the subunit level. Our kinetic analysis shows that the rate limiting step in the DcpS-catalyzed decapping reaction depends on the substrate concentration relative to the enzyme. When DcpS concentration is in excess of cap substrate, the decapping reaction rate is fast and the observed rate is limited at the initial substrate binding step. Under high substrate conditions, the rate limiting step is shifted to the conformational change/hydrolysis step (Table 1). We demonstrate that a dynamic conformational change of the N-
terminal domain relative to the C-terminal domain is essential for hydrolysis (Figure 3), confirming a mutually exclusive hydrolysis function between the two catalytic active sites.

An important mechanistic observation in these studies was that the rate limiting step of DcpS decapping changed from the initial substrate binding step to a subsequent conformational change/hydrolysis step when the amount of substrate was higher than DcpS (Figures 1 and 2). Therefore, at excess substrate concentrations, when both active sites of the DcpS dimer are occupied by substrate, we propose that the open to closed conformational change necessary for cap hydrolysis is slow (Figure 5). Such a negative allosteric regulation of DcpS is supported by several observations. First, the observed rate constant was reduced by 8 fold with wild type DcpS homodimer under high substrate conditions, while the DcpS$^{WT/BC}$ heterodimer with one wild type active site and a binding compromised active site, only displayed a 2 fold reduction of the rate constant (Table 2). Although we expected the DcpS$^{WT/BC}$ to be completely resistant to negative cooperativity rather than partially resistant, our experimental results indicate that the binding compromised monomer retains residual cap binding. This is not surprising considering the extensive network of amino acid contacts between DcpS and the cap structure (24). Additional multiple mutations to completely disrupt cap binding were not attempted since as indicated in Table 2 the decapping rate constant under single turnover conditions for the DcpS$^{WT/BC}$ was reduced relative to the wild type DcpS$^{WT/WT}$ protein. These data suggest that mutations in the cap binding are not well tolerated by the protein and further mutations were not tested.

Our data are consistent with an inherent negative cooperativity of the two active sites within DcpS as indicated by the cap bound co-crystal structure (16,24). A model was proposed whereby the N termini of both monomers within the DcpS dimer acts as a single inflexible domain that can alternate back and forth to form a hydrolysis competent closed and a nonproductive open conformations (24). This was expanded upon to include an intermediate state of both sites in the open confirmation (16,24). The high rigidity of the two intertwined N terminal domains prevents them from closing at the same time. Therefore, when an N terminus on a monomer closes, the one on the other is forced into an open position and only the closed form is active in decapping. The site in the open conformation is capable of cap substrate binding (24), but not its hydrolysis. Therefore, the DcpS dimer displays inherent negative cooperativity in decapping. Our results with the DcpS$^{WT/HIT}$ heterodimer provide experimental confirmation for the negative allosteric model where less then 2% decapping activity was detected under conditions of high substrate relative to enzyme (Figure 3D). Therefore, the biochemical analysis indicates that the WT site was not able to conform to a closed productive confirmation for hydrolysis when the HIT mutant site was bound and “locked” by the substrate. Our biochemical demonstration of cooperativity between the two active sites is consistent with a recent molecular dynamics simulation of the apo-form of DcpS which revealed that the protein dimer moves in a cooperative manner, where one side closes and the other side opens (30).

Collectively, our analysis of the heterodimers DcpS$^{WT/HIT}$ and DcpS$^{WT/BC}$ combined with the decapping kinetics of the wild type DcpS homodimer under both low and high substrates, as well as the known structural properties of DcpS suggest the following model of hydrolysis: under single turnover conditions, upon binding to the cap substrate at one binding site, the N terminus at this site closes at a fast rate for substrate cleavage, followed by release of the decapping products m$^3$Gp and ppN to complete a catalytic cycle (Figure 5A). Since the amount of substrate is lower than the enzyme, only one monomer is used per cycle. Therefore, there is no allosteric communication between the first and second monomer. The observed rate up to 200 nM DcpS concentration is limited by substrate binding. Under conditions where the substrate concentration is in excess of DcpS, both subunits are bound with the cap substrate. We propose that the conformational change from open to closed is required for decapping on either side and this conformational change is slow or unfavorable under excess substrate concentrations and consequently the observed rate of decapping is slower. After the substrate bound at the first site is hydrolyzed, the hydrolyzed products must be released from the active site and the vacant active
site is reoccupied by another substrate molecule or by the product. Therefore the same open to closed
conformational change limits the hydrolysis of cap
substrate at the second site. (Figure 5B). Although
less likely, the formal possibility exist that the
hydrolysis and release steps are slow or
unfavorable.

An interesting mechanism that regulates DcpS
proteins activity has been revealed in
Saccharomyces cerevisiae. This organism contains
two DcpS homologs, Dcs1p and Dcs2p with only
Dcs1p containing decapping activity (13). Dcs1p
and Dcs2p, are involved in a stress coping
mechanism (31). Under glucose-deprived
conditions, the catalytically inactive paralog,
Dcs2p, was shown to heterodimerize with
catalytically active Dcs1p and compromised its
substrate specificity and kcat (31,32). The
DcpS
WT/HIT
human protein heterodimer is
analogous to this situation with one active and one
inactive subunit and similarly led to a dramatic
decrease in decapping (Figure 3). There does not
appear to be a Dcs2p-like protein in human cells
suggesting that a heterodimer mediated inhibition
is unlikely, however our data indicates that
nonhydrolyzable substrates or compounds could
bind in one active site and inhibit decapping by
trapping the protein in an inactive state to inhibit
DcpS decapping.

DcpS has been characterized as an mRNA
decapping enzyme that is involved in mRNA
decay. Whether DcpS is involved in other cellular
processes is still an open question. The obvious
metabolic pathways DcpS may impact include
nucleotide biogenesis and catabolic pathways due
to its ability to hydrolyze cap dinucleotides. In
addition, DcpS activity may also impact earlier
steps of mRNA decay, as disruption of the yeast
Dcs1p decapping activity results in inhibition of 5´
to 3´ exonuclease activity (18). As shown in this
report, local increase of the substrate, would
inhibit DcpS decapping, which is analogous to the
disruption of Dcs1. Thus, substrate and product
inhibition of DcpS, which is part of the last step in
mRNA decay, could be a means to feedback and
regulate earlier steps in mRNA decay. Therefore,
the negative cooperative nature of DcpS activity
may provide a regulatory point for crosstalk
between both pathways. Studies are underway to
test this hypothesis.

In conclusion, our data provide evidence that
DcpS exhibits different enzymatic kinetics under
low and high substrate conditions. The presence
of negative cooperativity between two wild type
subunits, as well as the dramatically reduced
decapping activity displayed by the DcpS
WT/HIT
heterodimer under high substrate conditions
validate our previous dynamic decapping model
(24). Future studies to determine the local
concentrations of DcpS protein and cap
dinucleotide will begin to test the negative
allosteric regulatory model in cells.

Footnotes
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FIGURE LEGENDS

FIGURE 1. Decapping kinetics under conditions of excess DcpS relative to cap substrate. (A) Recombinant Flag-DcpS was overexpressed and purified from HEK293T cells and resolved by SDS-PAGE, followed by Sypro-Ruby staining. (B) Decapping reactions were carried out using monomer concentrations of 20nM, 50nM, 100nM, and 200nM DcpS and 10nM unlabeled cap structure substrate spiked with $^{32}$P labeled cap by a rapid quench-flow instrument at 25°C. The hydrolysis product and unhydrolyzed substrate were resolved by thin-layer chromatography (TLC) developed in 0.45 M (NH$_4$)$_2$SO$_4$ and exposed to a phosphor screen and developed on a PhosphorImager (see Materials and Methods). (C) The fraction of generated products was quantitated and plotted against the logarithmic value of the reaction time. The data points of each experiment were fit to a single exponential equation (Equation 1) to obtain the pseudofirst order decapping rate constants. The data represented here is an average of two sets of independent experiments carried out on two different days. The error bars indicate
the data range for each time point of the experiment. (D) The rate constants derived from (C) were plotted against DcpS concentrations. The slope of this linear plot gave the biomolecular rate constant for the cap hydrolysis. This value ranged between $2.3 \pm 0.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $6.8 \pm 0.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ based on four different data sets obtained through independent experimentation. (E) Amount of m7Gp product generated were calculated from (C) and plotted against DcpS concentrations. Even under the lowest enzyme concentration tested here (20nM), the reaction went to completion indicating that the enzyme exhibits a very tight binding for the substrate and has an equilibrium dissociation constant (K_a) of less than 20nM.

**FIGURE 2. Decapping kinetics under conditions of excess substrate relative to DcpS.** (A) 100 nM monomer concentration of DcpS was reacted with 200, 400, 800, 1600 nM cap substrate. The fraction of decapping product generated was plotted against the reaction time. The solid lines represent data points of each experiment fit to a single exponential equation (Equation 1). The data represented here is an average of three sets of independent experiments carried out on different days. The error bars indicate the data range for each time point of the experiment. (B) The pseudo first order decapping rate constants (initial rate nM s^{-1}/[DcpS]) were plotted against DcpS concentrations. The reaction rates appear to have saturated in the range of substrate concentrations used and implies a maximum rate constant of 0.0925 $\pm$ 0.003 s^{-1}.

**FIGURE 3. Trapping of cap substrate at the HIT mutant site of DcpS^{WT/HIT} prevents hydrolysis at the WT active site.** (A) A schematic representation of the double tagged DcpS^{WT/HIT} heterodimer is shown. The HIT site binds cap structure but is unable to hydrolyze it (24). (B) Recombinant DcpS^{WT/HIT} was overexpressed and purified from BL21-CodonPlus(DE3)-RIPL bacterial cells and resolved by SDS-PAGE, followed by Sypro-Ruby staining. Migration of the DcpS^{WT/HIT} heterodimer is indicated as is the copurifying bacterial GroEL whose identity was determined by mass spectrometry. (C) An EMSA was used to test the ability of DcpS^{WT/HIT} heterodimer to bind $^{32}$P-labeled cap analog. The DcpS^{WT/HIT} heterodimer containing two different tags that were used to selectively purify the protein possesses a Flag-tagged wild type protomer and a His-tagged HIT mutant protomer (lane 3). The His-tagged wild type homodimer was used in lane 2 and His-tagged HIT mutant homodimer was used in lane 4. The EMSA reactions were carried out with 1 $\mu$g of the indicated proteins and resolved on a 5.6% polyacrylamide gel. (D) The rapid quench-flow decapping assays of DcpS^{WT/HIT} were carried out under single and multiple turnover conditions and resolved by TLC. Single turnover conditions of 100 nM monomer concentration of enzyme and 10 nM substrate are denoted by the circles. Multiple turnover conditions of 100 nM monomer concentration of enzyme and 800 nM of substrate are indicated with the "x". The fraction of generated decapping product in each set of experiments were quantitated and plotted against the reaction times. The data points of the single turnover condition were fit to a biphasic, double exponential curve (Equation 2). The data represented here is an average of two sets of independent experiments carried out on different days. The error bars indicate the data range for each time point of the experiment. The maximal decapping rate of the multiple turnover experiment is lower than 2%.

**FIGURE 4. Both DcpS^{WT/WT} homodimer and DcpS^{WT/BC} heterodimer display decreased decapping rates under multiple turnover conditions.** (A) Schematic representations of the double tagged DcpS^{WT/WT} homodimer and DcpS^{WT/BC} heterodimer are shown. With the domain swapped nature of DcpS, DcpS^{WT/BC} contains a mutation in each protomer but upon heterodimerization the two mutations assemble at the same binding site rendering this site catalytically compromised. (B) Recombinant DcpS^{WT/WT} and DcpS^{WT/BC} were overexpressed and purified from BL21-CodonPlus(DE3)-RIPL bacterial cells and resolved by SDS-PAGE and visualized by Sypro-Ruby staining. (C) The DcpS^{BC/BC} homodimer has no detectable decapping activity with the decapping conditions employed. An in vitro decapping assay was carried out with the indicated monomer concentrations of DcpS^{BC/BC} homodimer and 200nM unlabeled cap structure spiked with $^{32}$P-labeled cap structure. The reactions were incubated for 30 sec at room temperature and terminated by 1.7N formic acid. The hydrolyzed product m7Gp and unhydrolyzed cap structure substrate were resolved by TLC and exposed to the phosphor screen and developed on a
FIGURE 5. Models of decapping mechanism under single (low substrate/enzyme ratio) and multiple (high substrate/enzyme ratio) turnover conditions. (A) Decapping model under conditions when substrate concentration is less than enzyme active sites displays no cooperativity between the two protomers. The ligand-free DcpS dimer exhibits a symmetric conformation or open conformation. Upon binding to the cap substrate at one binding site, the N-terminus at this site closes at a fast rate for substrate cleavage (closed conformation), followed by releasing of the decapping product m'Gp and ppN to complete a catalytic cycle. Only one protomer is used per cycle, therefore there is no allosteric communication between the first and second protomers. (B) DcpS hydrolysis of cap substrate displays negative cooperativity under conditions where substrate concentration is in excess of enzyme active sites. Under high substrate conditions, two substrates are bound to the dimer, and the conformational change involved in the closing of the N terminus on one of the substrates is either an unfavorable step or a slow limiting step. The rigidity of the domain swapped region forcing the N terminus of the second protomer to stay in an open position. After the substrate bound at the first site is hydrolyzed, the decapping products are released, and the N terminus of the second site closes at the same slow rate for hydrolysis followed by product release to complete the catalytic cycle.
A Flag DcpS

B  

Enzyme
20 nM
50 nM
100 nM
200 nM

Time (sec)
0.1
0.5
1
3

m\textsuperscript{7}Gp
m\textsuperscript{7}GpppG

D  

Rate Constant s\textsuperscript{-1}

E  

Produced m\textsuperscript{7}Gp (nM)

Liu et al. Figure 1
Liu et al. Figure 2

A

Produced mGp (nM)

Time (sec)

0 20 40 60 80 100 120

200nM Cap
400nM Cap
800nM Cap
1600nM Cap

100nM DcpS

B

Rate Constant s⁻¹

Substrate concentration (nM)

0 200 400 600 800 1000 1200 1400 1600

by guest on March 24, 2020
http://www.jbc.org/Downloaded from
A.

WT Protomer | HIT Protomer
---|---
Flag | His

No decapping

PpN
mGp

DcpS<sub>WT/HIT</sub>

B.

GroEL
His-DcpS<sup>WT</sup>
FLAG-DcpS<sup>WT</sup>

C.

DcpS

Cap only
His-WT/WT
Flag-WT/His-HIT
His-HIT/HIT

Protein-cap Complex

1 2 3 4

Cap

D.

Fraction Generated Product

Time (sec)

0 1 2 3 4

0.00 0.05 0.10 0.15 0.20 0.25 0.30

10nM Cap 100nM DcpS<sub>WT/HIT</sub>

Liu et al. Figure 3
Figure 4

(A) Schematic representation of the binding of DcpS proteins to GTP cap molecules. The WT protomer (left) shows normal binding, while the WT protomer with BC (right) shows compromised binding.

(B) Gel showing the binding of DcpS proteins to cap molecules. GroEL is used as a loading control.

(C) Quantitative analysis of cap binding to DcpS proteins. The fraction of cap generated is plotted against time for different concentrations of DcpS proteins.

(D) Graph showing the fraction of cap generated over time for 10nM and 800nM DcpSWT/BC.
| DcpS (nM)   | Low substrate | High substrate |
|------------|---------------|----------------|
|            | 10nM substrate | Substrate (nM) |
|            | 20            | 200            |
|            | 50            | 400            |
|            | 100           | 800            |
|            | 200           | 1600           |
| 20         | 200           | 9.25±0.038 nM/s | 9.25±0.038 nM/s |
| 50         | 400           | 8.95±0.068 nM/s | 8.95±0.068 nM/s |
| 100        | 800           | 9.55±0.099 nM/s | 9.55±0.099 nM/s |
| 200        | 1600          | 9.25±0.038 nM/s | 9.25±0.038 nM/s |

I = Initial rate, C = observed rate constant, R = fraction of maximal decapping product
|                    | Dcp$^{WT/WT}$ | Dcp$^{WT/BC}$ | Dcp$^{WT/HIT}$ |
|--------------------|--------------|--------------|---------------|
| **Enzyme 100 nM**  |              |              |               |
| **Substrate 10 nM**| C=0.735 ±0.037s$^{-1}$  
R= 0.96            | C=0.08±0.002s$^{-1}$    
R= 0.93                        | C=0.69 ±0.06s$^{-1}$    
R= 0.16                        |
| **Enzyme 100 nM**  | C=0.0955 ±0.0009s$^{-1}$  
R= 0.96            | C=0.042 ±0.0009s$^{-1}$    
R= 0.19                        | ND                          |
| **Substrate 800 nM**|              |              |               |
| **Rate constant**  |              |              |               |
| **change,**        |              |              |               |
| **Low substrate/ High substrate** | 7.7 fold | 1.9 fold | - |

C=observed rate constant, R=fraction of maximal decapping product
ND=not determined
Mechanistic and kinetic analysis of the DcpS scavenger decapping enzyme
Shin-Wu Liu, Vaishnavi Rajagopal, Smita S. Patel and Megerditch Kiledjian

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