INTRODUCTION

mRNA decay occurs in cytoplasmic regions of the cells, named P-bodies (processing bodies), consisting of many enzymes involved in mRNA turnover, including decapping enzymes [1,2]. The latter are required for the removal of the 5'-end cap of mRNAs. The human, plant and yeast decapping enzymes (Dcp2) exhibit a specific hydrolase activity, resulting in cleavage between the α- and β-phosphates of the m7GpppN cap to generate both m7GDP and monophosphorylated RNA products. Decapping enzymes have been found in humans, plants and yeasts, and have been discovered more recently in vaccinia virus (D10 protein). Although experimental evidences are lacking, three-metal- and two-metal-ion mechanisms have been proposed so far for the decapping enzymes. In the present study, we performed a biochemical characterization of the interaction of bivalent cations with the vaccinia virus D10 protein. Synergistic activation of the enzyme was observed in the presence of Mg2+ and Mn2+ ions, suggesting the existence of two metal-ion-binding sites on the D10 protein. Moreover, dual-ligand titration experiments using fluorescence spectroscopy demonstrated the presence of two metal-ion-binding sites on the enzyme. A three-dimensional structural model of the active site of the enzyme was generated which highlighted the importance of three glutamate residues involved in the co-ordination of two metal ions and a water molecule. Mutational analyses confirmed the role of two glutamate residues for the binding of metal ions. We demonstrate that one metal ion is co-ordinated by Glu132, while the second metal ion is co-ordinated by Glu144. Taken together, these results support the proposed two-metal-ion mechanistic model for the D10 decapping enzyme.

Key words: decapping enzyme, enzyme mechanism, mRNA metabolism, Nudix motif, structural modelling, vaccinia virus.
the complex to support co-ordination of the attacking nucleophile [18]. The precise mechanism used by the decapping enzymes has not yet been demonstrated. It has been suggested that these enzymes might utilize a three-bivalent-cation mechanism since the catalytic reaction is similar to the ADP-ribose pyrophosphatase, another Nudix hydrolase which harbours a three-metal-ion interaction in its crystal structure [19]. More recently, mutational analysis of AtDcp2p (Arabidopsis thaliana Dcp2 protein) led to the proposal of a two-metal-ion model for this enzyme [4].

The present study proposes a biochemical characterization of the interaction of bivalent cations with the vaccinia virus D10p. We investigated the ability of the enzyme to interact with both Mg$^{2+}$ and Mn$^{2+}$ ions and provide evidence for a two-metal-ion mechanism. Moreover, the generation of specific glutamate mutants of D10p outlined the residues involved in the interaction with the two metal ions.

MATERIALS AND METHODS

D10 expression and purification

A plasmid for the expression of a full-length D10 protein (248 amino acids) was generated by inserting the vaccinia D10R gene between the NdeI and HindIII cloning sites of the pET28a expression plasmid (Novagen). In this context, the D10 protein is fused in-frame with a N-terminal peptide containing six tandem histidine residues, and expression of the His$_6$-tagged protein is driven by a T7 RNA polymerase promoter. The resulting recombinant plasmid (pET-D10) was transformed into E. coli BL21(DE3) cells and a 1 litre culture of E. coli BL21(DE3)/pET-D10 was grown at 37 °C for 20 h. The cells were then harvested by centrifugation at 13 000 rev./min for 45 min at 4 °C.

Thawed bacteria pellets were resuspended in 50 ml of lysis buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl and 10% glycerol) containing 50, 100, 200, 500 or 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS/PAGE (12% polyacrylamide gel and visualized by UV-shadowing). The recombinant D10 protein was retained on the column and applied to a 2 ml column of Ni-NTA (Ni$^{2+}$-nitrilotriacetic acid)–agarose (Qiagen) that had been equilibrated with buffer A containing 200, 500 or 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS/PAGE (12% polyacrylamide gel) containing 50 μM and 10% sucrose, and cell lysis was achieved by adding lysozyme and Triton X-100 to final concentrations of 50 μg/ml and 0.1% respectively. The lysates were sonicated to reduce viscosity, and any insoluble material was removed by centrifugation at 13 000 rev./min for 45 min at 4 °C (Sorvall SLA15000 rotor). The soluble extract was applied to a 2 ml column of Ni-NTA (Ni$^{2+}$-nitrilotriacetic acid)–agarose (Qiagen) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with the same buffer and then eluted stepwise with buffer B (50 mM Tris/HCl, pH 8.0, 100 mM NaCl and 10% glycerol) containing 50, 100, 200, 500 or 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS/PAGE (12% gels). The recombinant D10 protein was retained on the column and recovered in the 500 and 1000 mM imidazole eluates. Following a 4 h dialysis against buffer C [50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 2 mM dithiothreitol (DTT) and 10% glycerol], the protein concentration was determined by the Bio-Rad dye-binding method using BSA as the standard. D10p E132A, E141A and E145A mutants were also cloned and expressed using this method.

7-Methyl-capped RNA synthesis

An RNA substrate of 81 nt was synthesized using a pair of complementary oligonucleotides including a T7 RNA promoter followed by the sequence of the substrate. Following transcription, the RNA substrate was purified on a denaturing 8% polyacrylamide gel and visualized by UV-shadowing. The corresponding band was excised, and then eluted from the gel by an overnight incubation in 0.1% SDS/0.5 M ammonium acetate. The RNA was then precipitated with propan-2-ol, and the pellets were resuspended in nanopure water. The purified 5′-triphosphorylated 81-nt RNA was processed further for 1 h in capping buffer (50 mM Tris/HCl, pH 7.5, 5 mM DTT and 20 mM MgCl$_2$) using the vaccinia virus RNA 5′-triphosphatase/RNA 5′-guanylyltransferase D1R to first obtain a diphosphorylated 5′-end. Afterwards, 1 μM [α-32P]GTP was added to the reaction mixture to allow the radiolabelled capping of the diphosphorylated RNA for 1 h. This capped RNA was again subjected to a phenol/chloroform purification and propan-2-ol precipitation. The resuspended capped RNA was methylated using the Saccharomyces cerevisiae guanine-7-methyltransferase (Abd1) in methylation buffer (50 mM Tris/HCl, pH 7.5, 5 mM DTT and 40 mM NaCl) with 2 μM adenosylmethionine for 1 h. This 7-methyl-capped RNA was again purified on an 8% polyacrylamide gel, excised and precipitated. The RNA substrate was then quantified by spectrophotometry and stored at −20 °C.

Decapping assays

Reaction mixtures (20 μl) containing 10 mM Tris/HCl (pH 7.5), 100 mM potassium acetate, 1 mM DTT, 0.5 μM γ-phosphate-labelled 7-methyl-capped RNA substrate and 1 μM purified D10 protein were incubated for 90 min at 37 °C in the presence of various metal ion concentrations. The reactions were quenched by adding 2 μl of 0.5 M EDTA. Aliquots of the mixtures were applied to a polyethyleneimine–cellulose TLC plate, which was developed with 0.5 mM LiCl and 1 M methanoic (formic) acid. The release of γ-phosphate-labelled [32P]GDP was revealed by autoradiography with a phosphomager (Amersham Biosciences) and quantified using ImageQuant 5.0 (Molecular Dynamics). In the case of NaF inhibition assays, the reactions were stopped after 1 h. Reaction mixtures where then treated as stated above. Furthermore, for the synergy assays, Mg$^{2+}$ and Mn$^{2+}$ ions were added separately or simultaneously to reaction mixtures, which were incubated for 1 h at 37 °C and treated further as stated above. Experiments were performed a minimum of three times separately.

Fluorescence measurements

Fluorescence was measured using an Hitachi F-2500 fluorescence spectrophotometer. Background emission was eliminated by subtracting the signal from either buffer alone or buffer containing the appropriate quantity of substrate. The extent to which ligands bind to the D10 protein was determined by monitoring the fluorescence emission of a fixed concentration of proteins and titrating with a given ligand. The binding can be described by eqn (1):

$$K_d = \frac{[\text{D10p}][\text{ligand}]}{[\text{D10p} \cdot \text{ligand}]}$$

(1)

where $K_d$ is the apparent dissociation constant, [D10p] is the concentration of the protein, [D10p- ligand] is the concentration of complexed protein, and [ligand] is the concentration of unbound ligand in solution. The proportion of ligand-bound protein, as described by eqn (1), is related to measured fluorescence emission intensity by eqn (2):

$$\Delta F/\Delta F_{\text{max}} = \frac{[\text{D10p} \cdot \text{ligand}]}{[\text{D10p}]_{\text{tot}}}$$

(2)
where $\Delta F$ is the magnitude of the difference between the observed fluorescence intensity at a given concentration of ligand and the fluorescence intensity in the absence of ligand, $\Delta F_{\text{max}}$ is the difference at infinite [ligand], and $[\text{D10p}]_{\text{tot}}$ is the total protein concentration.

If the total ligand concentration, $[\text{ligand}]_{\text{tot}}$, is in large molar excess relative to $[\text{D10p}]_{\text{tot}}$, then it can be assumed that [ligand] is approximately equal to $[\text{ligand}]_{\text{sat}}$. Eqns (1) and (2) can then be combined to give eqn (3):

$$\frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[\text{ligand}]_{\text{sat}}}{K_a + [\text{ligand}]_{\text{sat}}}$$

(3)

The $K_a$ values were determined from a non-linear least-square regression analysis of titration data by using eqn (3). Fluorescence experiments were performed a minimum of three times separately.

**Analysis of competitive metal ion binding**

Analysis of the effect of a fixed concentration of one metal ion ligand (iona) on the binding of a second ion ligand (ionb) was performed in a manner analogous to that reported previously for analysing the kinetics of a system in which two alternative substrates compete for the same enzyme-binding site [20]. The change in fluorescence ($\Delta F$) observed upon titration of D10p with iona in the presence of a fixed concentration of competing substrate (ionb) can be described by eqn (4):

$$\Delta F = \Delta F_{\text{max}(a)} \left( \frac{[\text{ion}_a]}{K_a} \right) + \Delta F_{\text{max}(b)} \left( \frac{[\text{ion}_b]}{K_b} \right)$$

$$+ [\text{ion}_a] + \frac{[\text{ion}_b]}{K_b}$$

(4)

where $\Delta F_{\text{max}(a)}$ and $\Delta F_{\text{max}(b)}$ are the changes in fluorescence produced at infinite concentrations of iona and ionb, respectively, and $K_a$ and $K_b$ are the apparent dissociation constants for iona and ionb, respectively. Eqn (4) was fitted to the simple ligand saturation isotherms for both iona and ionb.

**CD spectroscopy measurements**

CD measurements were performed using a Jasco J-810 spectropolarimeter. The samples were analysed in quartz cells with pathlengths of 1 mm. Far-UV wavelength scans were recorded from 200 to 250 nm. The average of three wavelength scans is presented. The ellipticity results were expressed as mean residue ellipticity, in millidegrees (mdeg).

**Homology modelling**

The crystal structure of SpDcp2p (Schizosaccharomyces pombe Dcp2 protein) was used as a template to model the vaccinia virus D10 protein. The atomic co-ordinates were obtained from the Protein Data Bank file 2A6T. The predicted three-dimensional structure of the D10 protein active site was generated with the Deep View program [21].

**RESULTS**

**Expression and decapping activity of D10p**

A His$_6$-tagged version of the 248-amino-acid vaccinia virus D10p was expressed in *E. coli* and purified by Ni-NTA–agarose chromatography. SDS/PAGE analysis demonstrated that the 31 kDa recombinant D10p was the predominant polypeptide in the purified fraction (Figure 1A). Size-exclusion chromatography assays performed on the purified D10p to assess its possible homodimerization revealed a unique absorbance peak around 31 kDa, the expected molecular mass of a D10p monomer (results not shown).

The decapping activity of the purified protein was initially evaluated using a radiolabelled methyl-capped RNA substrate in the presence of increasing concentrations of Mn$^{2+}$ ions (Figure 1B). The reactions products were then separated by TLC. As seen in Figure 1B, only the $^5$GDP product and the initial capped $^6$Gppp-RNA substrate were detected, indicating that the reaction is specific and does not yield any other side product (Figure 1B). Decapping activity reached a maximum ($>$95%) at a concentration of 5 mM for Mn$^{2+}$ (Figures 1B and 1D).

We next sought to investigate the ability of Mg$^{2+}$ ions to support the decapping activity of the vaccinia virus D10 protein. Once again, maximal hydrolysis was reached at a metal ion concentration of 5 mM (Figure 1C). However, under these conditions, only 45% of the radiolabelled methyl-capped RNA substrate was hydrolysed by the enzyme (Figures 1C and 1D). This difference in catalysis is also reflected by the different pseudo-catalytic constants ($k_{cat}$ determined in the presence of the two metal ions (0.2 min$^{-1}$ and 0.1 min$^{-1}$ for Mn$^{2+}$ and Mg$^{2+}$ ions respectively).

Previous studies have demonstrated the high sensitivity of other Nudix hydrolases, such as Ap4P and the coenzyme A hydrolyase, to F$^-$ ions, whereas AtDcp2p was only moderately affected under the same conditions [4,14,22]. The susceptibility of the recombinant D10p to inhibition by F$^-$ ions was therefore tested. Similarly to AtDcp2p, D10p did not present any susceptibility to F$^-$ ions in the presence of MnCl$_2$ or MgCl$_2$ (Figure 1E). This effect is also evident by the $k_{cat}$ values which remained identical upon incubation with 0.1 mM NaF (0.2 min$^{-1}$ and 0.1 min$^{-1}$ in the presence of Mn$^{2+}$ and Mg$^{2+}$ ions respectively). However, a moderate inhibition could be observed in the presence of MgCl$_2$ when the enzyme was incubated with a very high concentration of NaF (5 mM) (Figure 1E). These results demonstrate that the two decapping enzymes, D10p and AtDcp2p, show a similar lack of inhibition by F$^-$ ions, which sets them apart from other Nudix hydrolase family members.

**Interaction with bivalent cations**

Fluorescence spectroscopy was used to monitor the interaction of metal ions to D10p. Using the intrinsic fluorescence of the sole tryptophan residue of the D10 protein (Trp$^3$), the enzyme was excited at a wavelength of 290 nm. The enzyme in standard buffer generated a fluorescence emission spectrum with a maximal peak at 336 nm (Figure 2A). Analysis of the molar intensity of the fluorescence emission of D10p revealed a linear change of 7.3 fluorescence intensity units/μM of protein over the range examined (Figure 2A, inset). This relatively small change can probably be attributed to small losses of proteins from solution through adhesion. All subsequent binding experiments were performed at a protein concentration of 1 μM, with the assumption that the binding equilibrium was not affected by the presence of aggregation equilibrium.

To evaluate the $K_a$ for the interaction of metal ions with the recombinant D10p, various concentrations of either MgCl$_2$ or MnCl$_2$ were added to the purified enzyme and the fluorescence emission was monitored. A decrease in fluorescence intensity was observed when the protein was titrated with increasing amounts of Mg$^{2+}$ or Mn$^{2+}$ ions (Figure 2B, inset). No variation in fluorescence intensity was observed upon addition of Ca$^{2+}$, Na$^+$ or Li$^+$ ions to D10p, supporting the specificity of the binding observed...
in the presence of Mg$^{2+}$ or Mn$^{2+}$ ions (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200027add.htm). The binding data for Mg$^{2+}$ and Mn$^{2+}$ ions indicate that the enzyme displays similar affinity for both metal ions, as determined from the respective $K_d$ and $\Delta G$ values (Figure 2B and Table 1). In order to confirm these results, we also evaluated the binding of Mn$^{2+}$ and Mg$^{2+}$ to D10p using CD. The molar ellipticity of the enzyme was monitored from 200 to 250 nm as a function of added Mg$^{2+}$ or Mn$^{2+}$ ions (Figure 2C). A plot of the ellipticity variation at 222 nm, representative of changes in $\alpha$-helical structures, as a function of added bivalent cations is presented (Figure 2D). The estimated dissociation constants corroborate the results obtained in fluorescence spectroscopy experiments, with $K_d$ values of 4.7 ± 1.7 mM for the binding of Mn$^{2+}$ ions, and 2.1 ± 0.4 mM for the interaction of Mg$^{2+}$ ions with D10p.

Analysis of Hill plots generated from the Mg$^{2+}$ and Mn$^{2+}$ fluorescence spectroscopy binding assays indicated a lack of co-operativity ($h = 0.9 ± 0.3$) for the binding of both ions to D10p (see Supplementary Figure S2A at http://www.BiochemJ.org/bj/420/bj4200027add.htm). However, derivation of the binding data with the Scatchard equation yielded curves displaying the typical appearance of two-site binding plots, for both metal ions (Supplementary Figure S2B). These results suggest the presence of two metal-ion-binding sites on D10p with no discernable co-operativity between the two sites. The association rate of both ions to D10p was also evaluated by real-time fluorescence spectroscopy (Figure 2E). Both ions associate readily with the enzyme, as judged from the association rate values obtained for Mn$^{2+}$ and Mg$^{2+}$ binding to D10p (4.1 ± 0.4 and $3.7 ± 0.7$ mM$^{-1} \cdot $s$^{-1}$ respectively).

With the difference in catalytic activity of D10p observed in the presence of Mg$^{2+}$ or Mn$^{2+}$ ions, and the differences in the binding of the two ions to the enzyme, we wondered whether the two metal ions bind to the same site on D10p. To answer this question, we performed competitive alternative ligand-binding experiments using fluorescence spectroscopy. The saturation isotherm determined for the binding of Mn$^{2+}$ to D10p was compared with curves obtained in the presence of Mn$^{2+}$ and either 1 or 5 mM Mg$^{2+}$ (Figure 2F). We also performed the reverse experiment, where Mg$^{2+}$ binding was competed with increasing Mn$^{2+}$ concentrations (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/420/bj4200027add.htm). Both results are in accordance with a model in which the binding of Mg$^{2+}$ and Mn$^{2+}$ is not mutually exclusive [23,24]. Instead, the addition of the second ion (Mg$^{2+}$) yields an additive variation in the fluorescence emitted by D10p already saturated with the first one. We conclude

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that at least two metal-ion-binding sites are present on the enzyme.

Synergistic activation of D10p by metal ions
The previous results led us to suspect that D10p exploits a two-metal-ion mechanism for catalytic activity to occur. With the assumption that each site demonstrates a preference for either Mg\(^{2+}\) or Mn\(^{2+}\), a mixture of suboptimal concentrations of each metal ion should produce a synergistic activation of the D10p activity. Such activation was observed using concentrations of 0.25 and 0.5 mM of each ion (Figure 3). In the presence of 0.25 mM MgCl\(_2\), the addition of D10p resulted in the release of 0.11 \(\pm\) 0.05 pmol of product, whereas 1.4 \(\pm\) 0.5 pmol was generated in the presence of 0.25 mM MnCl\(_2\). However, with 0.25 mM of both ions, 3.1 \(\pm\) 0.2 pmol of product was released. The latter product formation equals to more than the sum of the products generated with each ion separately. Similar results were obtained using 0.5 mM MgCl\(_2\) or MnCl\(_2\). This synergistic activation of D10p is in agreement with a two-metal mechanism [25,26].

In order to emphasize that the previous results were specifically due to the presence of these two particular ions, and not of an ionic environment change generated by the addition of another bivalent cation in solution, the latter experiment was repeated in the presence of CaCl\(_2\) and CoCl\(_2\). Ca\(^{2+}\) ions could not support D10p activity and did not produce a synergistic activation of D10p in the presence of Mn\(^{2+}\) (Figure 3). The Co\(^{2+}\) ions, however,

### Table 1  Bivalent cation dissociation constants (\(K_d\)) and Gibbs free energy (\(\Delta G\))

<table>
<thead>
<tr>
<th></th>
<th>(K_{D2^+}) (mM)</th>
<th>(K_{D3^+}) (mM)</th>
<th>(\Delta G_{D2^+}) (kcal/mol)</th>
<th>(\Delta G_{D3^+}) (kcal/mol)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>2.0 (\pm) 0.6</td>
<td>4.2 (\pm) 1.3</td>
<td>(\sim) 3.7 (\pm) 0.2</td>
<td>(\sim) 3.4 (\pm) 0.2</td>
</tr>
<tr>
<td>E132A</td>
<td>9.1 (\pm) 2.2</td>
<td>7.6 (\pm) 2.2</td>
<td>(\sim) 2.8 (\pm) 0.1</td>
<td>(\sim) 2.9 (\pm) 0.2</td>
</tr>
<tr>
<td>E141A</td>
<td>2.5 (\pm) 0.8</td>
<td>9.0 (\pm) 2.0</td>
<td>(\sim) 3.6 (\pm) 0.2</td>
<td>(\sim) 2.8 (\pm) 0.1</td>
</tr>
<tr>
<td>E145A</td>
<td>5.1 (\pm) 2.1</td>
<td>25.9 (\pm) 5.9</td>
<td>(\sim) 3.2 (\pm) 0.3</td>
<td>(\sim) 2.2 (\pm) 0.1</td>
</tr>
</tbody>
</table>

1 kcal = 4.184 kJ.
Synergistic activation of D10p

Figure 3

Reaction mixtures containing 1 μM D10p, 0.5 μM capped RNA substrate and various concentrations of metal ions, were incubated for 1 h at 37°C. The products were analysed by TLC and quantified by autoradiography. Results are means ± S.E.M. of three replicates.

Supported D10p catalytic activity, but also failed to demonstrate a synergistic effect on the enzyme when tested with Mn²⁺ ions (Figure 3). We therefore conclude that the synergistic activation of D10p by Mn²⁺ and Mg²⁺ is a specific effect, due to the presence of both ions and not to a modification of the ionic strength of the reaction mixture.

Modelling of the active site and mechanism of action

The Dcp2 proteins examined so far present three highly conserved domains required for different aspects of their activity. A main 109-amino-acid Nudix domain comprising the 23-amino-acid Nudix motif is required for the hydrolytic activity of the enzymes. This region is surrounded by two additional domains (a 38-amino-acid Box A and a 20-amino-acid Box B) [3,15]. Box A has been associated with the specificity of the hydrolytic activity of the enzyme in hDcp2p (human Dcp2 protein), whereas Box B is structured into an α-helix and appears to be required for the interaction with RNA. Analysis of the D10p amino acid sequence does not reveal a high homology with the Box A found in other decapping enzymes. Only four of the 14 absolutely conserved amino acids in yeast, plants and humans are found in D10p (Figure 4A) [6]. Moreover, the enzyme possesses only two of the four main amino acids conserved in the Box B of decapping enzymes (Figure 4A). However, structural modelling of the D10p region corresponding to the Box B suggests that it could still adopt an α-helical structure (results not shown). Interestingly,

Figure 4

Sequence conservation within the Nudix motif of decapping enzymes and modelling of the D10 protein

(A) Alignments of amino acids 11–48, 126–148 and 216–235 respectively from the Box A, Nudix motif and Box B of the vaccinia virus D10 protein are presented with the corresponding motifs in AtDcp2, S. cerevisiae (ScDcp2), hDcp2 and SpDcp2. The positions of amino acids corresponding to the sequence of the vaccinia virus D10 protein are indicated. Black boxes represent consensus amino acids in all five sequences, whereas grey boxes highlight conserved amino acids. The Nudix motif consensus sequence is presented below the alignment. (B) Predicted three-dimensional structure of the active site of the vaccinia virus D10p (amino acids 111–123). The characteristic loop–α-helix–loop fold found in other decapping enzymes is shown in a darker shade of grey. (C) Mechanistic model for the two-metal-ion mechanism of the D10p decapping activity. In the chemical step, Glu132 and Glu145 are engaged in the co-ordination of bivalent cations, whereas Glu141 would be required for the polarization of the attacking water molecule involved in catalysis.

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Two-metal-ion mechanism for a decapping enzyme

Figure 5  Effect of D10p glutamate mutants on activity and ion interaction

(A) The wild-type (wt) and D10p mutants (E132A, E141A and E145A) were incubated with a capped RNA substrate (0.5 μM) for 1.5 h at 37 °C with 10 mM MgCl₂ and 10 mM MnCl₂ in decapping buffer. The reactions were stopped by the addition of EDTA to 50 mM. The products were analysed by TLC, quantified by autoradiography, and are presented as a percentage of the wild-type activity.

(B–E) Hanes–Wolf representation of the saturation isotherms of the fluorescence emission spectra for the binding of Mg²⁺ (B and D) and Mn²⁺ ions (C and E) to the wild-type D10p (■) and mutant D10p: E132A (□), E141A (○) and E145A (△). (F) Hanes–Wolf representation of the saturation isotherms of the CD far-UV spectra for the Mn²⁺ ion binding by wild-type D10p (■) and mutant D10p: E141A (○) and E145A (△).

we also observed that the D10p Nudix motif presents a very high conservation with the Nudix motif from the Dcp2p of other species (Figure 4A). Even though D10p has a low overall identity with the decapping enzymes presented in Figure 4(A), with values ranging from 18 % for hDcp2p and 22 % for the AtDcp2p, structural alignment of part of the Nudix domain of D10p with SpDcp2p showed a high level of homology between the catalytic centres of both enzymes. Amino acids 111–123 of D10p were therefore modelled based on the crystal structure of SpDcp2p (Figure 4B). The loop-α-helix–loop structural motif is conserved in D10p, along with the three critical glutamate residues (Glu¹⁴¹, Glu¹⁴⁴ and Glu¹⁴⁵) of the Nudix motif.

On the basis of this structural model, sequence alignments and previously suggested mechanisms [4,12,27], a model of the catalytic step of the decapping mechanism of D10p was produced (Figure 4C). In this model, a two-metal-ion mechanism is proposed for the chemical step of the reaction, where Glu¹⁴¹ is involved in polarization and positioning of a water molecule for the attack on the α–β-phosphate bond, whereas Glu¹³² and Glu¹⁴⁵ both co-ordinate a bivalent cation. The Glu¹⁴⁵-co-ordinated ion (Me²⁺₁) would assume a role in stabilizing the water for catalysis, whereas the Glu¹³²-co-ordinated ion (Me²⁺₂) would sustain a structural role by stabilizing the interaction with RNA.

Mutational analysis

In order to provide experimental evidence for the proposed mechanism, we generated a series of enzymatic mutants. Glu¹³², Glu¹⁴¹ and Glu¹⁴⁵ were replaced by alanine, and the mutant poly-peptides were expressed and purified in parallel with the wild-type enzyme. The relative activity of the purified mutants was assessed and compared with the wild-type D10p. Activity levels of 7.8 ± 0.2, 10.0 ± 1.0 and 12.2 ± 2.0 % were obtained for the E132A, E141A and E145A mutants respectively (Figure 5A). Furthermore, fluorescence spectroscopy was used to evaluate the ability of the mutants to bind Mg²⁺ and Mn²⁺ ions, in order to investigate a possible correlation between the lack of activity of the mutants and a decrease in their affinity for ions (Figures 5B–5E and Table 1). The E132A mutant showed a 5-fold reduced affinity for Mg²⁺, but retained a Mn²⁺ affinity similar to that of the wild-type. The E141A mutant demonstrated only slight variations in both Mg²⁺ and Mn²⁺ binding, as is evident from the $K_d$ and $\Delta G$ values. Finally, the E145A mutant also presented a slight 2-fold variation in its affinity for Mg²⁺, whereas the Mn²⁺ binding was reduced 6-fold. In the case of the E132A and E145A mutants, only one proposed metal-ion-binding site was tentatively disrupted by the single point mutations, leaving a second possible binding site available for interaction. This probably explains the low variation obtained for the dissociation constants.

The interaction of Mn²⁺ with the D10p E141A and E145A mutants was also confirmed using CD. The dissociation constants obtained are similar to those measured using fluorescence spectroscopy, with values of 6.4 ± 2.3 and 18.5 ± 5.8 mM for the titration of D10p E141A and E145A with Mn²⁺ ions respectively (Figure 5F). These results demonstrate that the mutations affect the binding of bivalent cations by the enzyme in ways that correlate with our proposed mechanism. It also points out a preference for Mg²⁺ at the Glu¹³² binding site, and a preference for Mn²⁺ at the Glu¹⁴⁵ binding site.
DISCUSSION

To date, vaccinia virus represents the only mammalian virus for which decapping enzymes have been experimentally demonstrated [8–10]. The decapping activity appears to be critical to accelerate viral mRNA turnover and to eliminate competing cellular mRNAs. In the present study, the interaction of the vaccinia virus D10 decapping enzyme with bivalent cations was evaluated. According to our results, a synergistic activation of the D10p catalytic activity is observed when both Mg\(^{2+}\) and Mn\(^{2+}\) ions are present. Moreover, competitive alternative ligand-binding assays confirmed the presence of two metal-ion-binding sites on the enzyme. Our mutational analysis also revealed a preference by specific amino acids for a particular ion. Namely, we observed a decrease in Mg\(^{2+}\) ion binding when Glu\(^{132}\) was replaced by alanine and a reduced affinity for Mn\(^{2+}\) ions for the E145A mutant. These results support a two-metal-ion mechanism with the enzyme being able to catalyse its hydrolytic reaction in the presence of either bivalent ions, but with a higher activity when both sites are filled with their preferential ion. In our model, one of the metal ions (Me\(^{2+}\)) is co-ordinated by Glu\(^{132}\) and is involved in contacts with the β- and γ-phosphates of the cap, stabilizing the \(^{16}\text{GDP}\) leaving group, and probably involved in the positioning of the methylated cap for the hydrolytic attack. The other metal ion (Me\(^{2+}\)) is co-ordinated mainly by Glu\(^{145}\) and is likely to be required for the orientation of the water molecule for the attack on the α-β-phosphate bond of the cap structure. Glu\(^{141}\) is also likely to be at hydrogen-bonding distance of Me\(^{2+}\), and could be involved in its co-ordination, especially after the catalytic step, as seen in other Nudix hydrolases [12]. During the catalytic step, Glu\(^{141}\) would serve as a general base to either initiate catalysis or stabilize the attacking water molecule [12].

The mechanism described here for the D10 viral decapping enzyme could apply to other decapping enzymes in metazoans. However, analysis of the D10p sequence showed only partial homology with Dcp2 proteins. Although the Nudix motif is absolutely conserved, the Boxes A and B commonly found in Dcp2p are virtually absent from D10p. The protein, with absolutely conserved, the Boxes A and B commonly found in Dcp2p, possess an absolute catalytic activity. D10p activity or its localization in cells [31]. Moreover, the Ge-1 cellular protein could be investigated for possible interactions with D10p since it has been shown to localize to the P-bodies and interact with host Dcp enzymes [34–36]. Further analysis could lead to the discovery of cellular interaction partners with D10p and assess its possible localization to the mRNA processing bodies in infected cells.

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SUPPLEMENTARY ONLINE DATA
Characterization of the vaccinia virus D10 decapping enzyme provides evidence for a two-metal-ion mechanism

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Figure S1 Specificity of metal ion binding to D10p
Fluorescence emission spectra of 1 μM D10p in decapping buffer. Excitation was performed at 290 nm, and the emission spectrum was scanned from 310 to 440 nm. The fluorescence emission of D10p is monitored in the absence (■) or in the presence of 50 mM Ca\(^{2+}\) (○), Na\(^{+}\) (▲) or Li\(^{+}\) (▼) ions.

Figure S2 Hill and Scatchard derivations for metal ion binding to D10p
Derivation of the binding data from fluorescence spectroscopy binding experiments with the Hill or Scatchard equations. (A) Hill plots for Mn\(^{2+}\) (■) and Mg\(^{2+}\) (▲) binding to D10p, and Scatchard plots for the interaction of Mn\(^{2+}\) (B) and Mg\(^{2+}\) (C) with the enzyme.

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Figure S3  Competitive alternative ligand-binding experiment

Standard titration assays were performed using Mg$^{2+}$ ions in the presence of increasing amounts of Mn$^{2+}$ ions. The concentrations of Mn$^{2+}$ ions used in these experiments were 0 (■), 1 (○) and 5 (□) mM.