Structure and enzymatic mechanism of a moonlighting dUTPase

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Structure and enzymatic mechanism of a moonlighting dUTPase

Genome integrity requires well controlled cellular pools of nucleotides. dUTPases are responsible for regulating cellular dUTP levels and providing dUMP for dTTP biosynthesis. In Staphylococcus, phage dUTPases are also suggested to be involved in a moonlighting function regulating the expression of pathogenicity-island genes. Staphylococcal phage trimeric dUTPase sequences include a specific insertion that is not found in other organisms. Here, a 2.1 Å resolution three-dimensional structure of a ϕ11 phage dUTPase trimer with complete localization of the phage-specific insert, which folds into a small β-sheet mini-domain reaching out from the dUTPase core surface, is presented. The insert mini-domains jointly coordinate a single Mg²⁺ ion per trimer at the entrance to the threefold inner channel. Structural results provide an explanation for the role of Asp95, which is suggested to have functional significance in the moonlighting activity, as the metal-ion-coordinating moiety potentially involved in correct positioning of the insert. Enzyme-kinetics studies of wild-type and mutant constructs show that the insert has no major role in dUTP binding or cleavage and provide a description of the elementary steps (fast binding of substrate and release of product). In conclusion, the structural and kinetic data allow insights into both the phage-specific characteristics and the generally conserved traits of ϕ11 phage dUTPase.

1. Introduction

Genome integrity ultimately depends on dedicated mechanisms of DNA damage recognition and repair, as well as the regulation of cellular nucleotide pools to allow the synthesis of DNA with high fidelity (Langerak & Russell, 2012; Rouse & Jackson, 2002; Niida et al., 2010). Although the core pathways are well conserved from bacteria to lower and higher eukaryotes, the set of protein macromolecules involved in these processes varies among different evolutionary branches, reflecting the adaptation of the organisms to cellular and environmental conditions. Viruses present a specific group from this point of view: while importantly relying on the set of genes and proteins endogenous to the host organism, viruses also encode several key factors in their own genome that interact with the DNA-repair apparatus of the host cell (Chaurushiya & Weitzman, 2009).

A frequently occurring mistake in DNA is the presence of uracil, either from cytosine deamination or thymine-replacing uracil incorporation (Vértessy & Töth, 2009; Castillo-Acosta et al., 2012; Wilson et al., 2012; Galperin et al., 2006). Two enzyme families, uracil-DNA glycosylases and dUTPases, are involved in eliminating or preventing the incorporation of erroneous uracil, respectively (Doseth et al., 2012; Visnes et al., 2009; Pecsi et al., 2012; Muha et al., 2012). Several viruses encode
representatives of both of these families, and it has been shown that viral genes for these enzymes have significant roles in the efficiency of the virus in invading host cells, especially when the expression of the respective host genes is down-regulated (e.g. in resting or differentiated cells; Payne & Elder, 2001). The dUTPase enzyme family constitutes trimeric, dimeric and monomeric enzymes, where the trimeric and monomeric representatives both show a well conserved β-pleated jelly-roll fold and five conserved motifs involved in enzyme activity (Vértessy & Tóth, 2009; Persson et al., 2001; Freeman et al., 2004; Tarbouriech et al., 2005). Dimeric dUTPases belong to another protein family with no structural similarities (Moroz et al., 2004; Harkiolaki et al., 2004).

Interestingly, annotated genome databases of staphylococcal phages report that phages may encode dUTPase genes either from the trimeric or the dimeric family, depending on the specific phage. It was recently shown that staphylococcal phage dUTPases may be responsible for inducing horizontal gene transfer of the mobile genetic elements termed staphylococcal pathogenicity islands (SAPIs; Tormo-Má's et al., 2010). SAPIs are encoded within the bacterial genome and are under the regulation of repressor proteins (Stls) specific for the different SAPIs (Novick & Subedi, 2007; Ruizin et al., 2001, Tormo et al., 2008). As such, the Stl repressor of SAPIbov1 down-regulates the expression of the proteins responsible for SAPIbov1 mobilization. Helper phages are already known to relieve this repression and to induce the excision and replication of SAPIs (Maïques et al., 2007). The dUTPases encoded within the helper phages have been implicated in this task (Tormo-Má's et al., 2010). An in-depth structural and functional characterization of phage dUTPases is indispensable to understand this biological phenomenon of de-repression, which is also important from a biomedical point of view since SAPIs encode virulence factors and toxins and their spreading among different strains is a major biomedical challenge (Novick & Subedi, 2007). A recent study reported three-dimensional structures of the 80k phage dUTPase and its complexes at 2.8–3.1 Å resolution. These structures provided reliable information on the trimeric protein fold but have not yet allowed full insight into protein–ligand interactions (Tormo-Má's et al., 2013). It has also been reported that the φ11 phage dUTPase (see Fig. 1 and Supplementary Fig. S2 for sequence alignments) is much more efficient in de-repression compared with the 80k phage dUTPase, making it an interesting target for detailed investigations (Tormo-Má's et al., 2010, 2013).

In the present work, we describe the structure of the φ11 phage trimeric dUTPase at 2.1 Å resolution using X-ray crystallography and also characterize its enzyme-kinetic cycle. We analyze the phage-specific structural characteristics, compare it with the 80k phage dUTPase structure and show that the phage-specific insert forms four short antiparallel β-strands in antiparallel organization. The orientation of this mini-domain is aided by coordination of a Mg²⁺ ion, accommodated by one aspartate residue from the insert in each of the three subunits. Using fluorescence spectroscopy with different site-specific tryptophan probes, as well as steady-state and transient kinetics, we provide a description of the catalytic cycle of the phage dUTPase.

2. Materials and methods

2.1. Cloning, mutagenesis, expression and purification of recombinant proteins

The cDNA of wild-type φ11 phage dUTPase was amplified from an expression vector previously described in Leveles et al. (2011) and was cloned into the EcoRI/XhoI restriction sites of the pGEX-4T-1 vector in frame with the N-terminal glutathione-S-transferase tag, as well as with a thrombin cleavage site and a 6×His tag between the glutathione-S-transferase tag and the dUTPase sequence. This fusion protein –ligand interactions (Tormo-Má's, 2007).
construct was expressed using the *Escherichia coli* BL21 (DE3) Rosetta expression system. 0.51 LB medium was inoculated with Rosetta cells from a 5 ml overnight culture and grown at 310 K until the OD₆₀₀ reached 0.4. After 30 min cooling at 303 K, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 303 K. The cells were harvested by centrifugation at 4000 g for 20 min and stored at 193 K. Thawed cells were resuspended in 15 ml 1× PBS buffer pH 7.3 containing 200 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 3 μg ml⁻¹ RNase, 3 μg ml⁻¹ DNase and EDTA-free Complete ULTRA protease-inhibitor preparation (Roche, Switzerland). The solution was sonicated and centrifuged and the supernatant was applied onto a pre-equilibrated PBS buffer pH 7.3 containing 200 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 3 μg ml⁻¹ RNase, 3 μg ml⁻¹ DNase and EDTA-free Complete ULTRA protease-inhibitor preparation (Roche, Switzerland). The solution was sonicated and centrifuged and the supernatant was applied onto a pre-equilibrated benchtop glutathione-agarose affinity-chromatography column. After washing the column with ten volumes of buffer, the dUTPase was eluted using overnight thrombin cleavage to remove the glutathione-S-transferase tag, resulting in protein preparations of >95% homogeneity as judged by SDS–PAGE.

This construct contained a GSPEFHHHHHHGS N-terminal extension preceding the original dUTPase sequence. We checked that this extension did not change the dUTPase enzymatic activity compared with the previously investigated tag-free dUTPase (Leveles *et al.*, 2011) and will use the abbreviation φ11DUT WT to refer to it in this study. The molecular mass of the φ11DUT WT protein is 19 853 Da as calculated from the amino-acid sequence. Throughout this study, the numbering of the dUTPase sequence is according to the original cDNA (Uniprot ID Q8SDV3), starting with the physiologically encoded initial methionine.

The following mutants were created by QuickChange site-directed mutagenesis (Stratagene) using suitable mutagenic primers (listed in Supplementary Table S1): φ11DUT F108W, φ11DUT F164W, φ11DUT E183STOP and φ11DUT A101–122. All constructs were verified by DNA sequencing of both strands. Mutants were expressed and purified as for the wild-type proteins. All protein preparations were >95% pure as judged by SDS–PAGE.

Protein concentrations were determined using Bradford’s assay or by UV spectrometry using $A_{280}$ values of 0.79, 1.08, 1.08, 0.84 and 0.81 cm⁻¹ for the wild type and the φ11DUT F108W, φ11DUT F164W, φ11DUT E183STOP and φ11DUT A101–122 mutants, respectively, as calculated from the amino-acid sequence. Concentrations are given in subunits (molecular-mass values were calculated based on amino-acid sequence).

### 2.2. Crystal structure determination

Crystals of φ11 phage dUTPase in the presence of 1 mM $\alpha,\beta$-imido-dUTP and 5 mM Mg²⁺ were grown by the hanging-drop vapour-diffusion method at 293 K as described previously (Leveles *et al.*, 2011). A diffraction data set was collected from a single crystal in a nitrogen cryostream at 100 K on the PXIII beamline of the Swiss Light Source, Villigen, Switzerland (PILATUS 2M detector, wavelength 1.00 Å, φ range of 180° with an increment of 0.1°; crystal-to-detector distance 210 mm). Data were processed to 2.1 Å resolution using the XDS and XSCELE programs (Kabsch, 2010). The data-collection statistics are summarized in Table 1.

The structure was solved by molecular replacement using MOLREP v.0.2.35 (Vagin & Teplyakov, 2010) from the CCP4 package (Winn *et al.*, 2011). The structure of mycobacterial dUTPase (PDB entry 3hza; Pecsi *et al.*, 2010) was used as a model for molecular replacement for a different crystal form of φ11 dUTPase (Leveles *et al.*, 2011; space group I23, resolution 2.98 Å, one dUTPase subunit in the asymmetric unit); however, the insertion loop could not be unambiguously built into electron density. The crystallographic dUTPase trimer generated from this partially built low-resolution structure was used as a model for molecular replacement of the present structure. The asymmetric unit contains six subunits (two dUTPase trimers); the Matthews coefficient was 2.31 Å³ Da⁻¹.

Model building was carried out with Coot v0.6.2.2 (Emsley *et al.*, 2010) using graphical comparison of noncrystallographically related subunits (NCS ghost control option). The model was refined by likelihood-based refinement using REFMAC v.5.6.0117 (Murshudov *et al.*, 2011), in which TLS refinement (Winn *et al.*, 2001) was carried out for quaternary-structure regions (six TLS groups each consisting of core residues of one subunit, the C-terminal arm region of the next subunit interacting with these and Mg²⁺–$\alpha,\beta$-imido-dUTP

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**Table 1**

<table>
<thead>
<tr>
<th>Crystallographic data-collection and refinement statistics.</th>
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<tr>
<td><strong>Data collection</strong></td>
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<tr>
<td>Space group</td>
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<tr>
<td>Unit-cell parameters (Å)</td>
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<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>Total No. of reflections</td>
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<td>No. of unique reflections</td>
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<tr>
<td>Multiplicity</td>
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<tr>
<td>Completeness (%)</td>
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| **Refinement**                                            |
| No. of dUTPase subunits in asymmetric unit                |
| No. of protein atoms                                      |
| No. of ligand atoms                                       |
| No. of waters                                             |
| No. of Mg²⁺ ions                                          |
| $R_{	ext{merge}}$                                         |
| Luzzati plot coordinate error (Å)                         |
| Average B factors (Å²)                                    |
| Wilson factor                                             |
| Protein atoms                                            |
| Ligand atoms                                             |
| Water                                                     |
| Mg²⁺ ions                                                 |

| **Ramachandran plot analysis§, residues in (%)**          |
| Favoured region                                           |
| Allowed region                                            |
| Disallowed region                                         |

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| Values in parentheses are for the outer shell. |

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| Values in parentheses are for the outer shell. |

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| Values in parentheses are for the outer shell. |

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| Values in parentheses are for the outer shell. |
bound at the active site); noncrystallographic restraints were introduced for regions with similar conformations of each subunit within both trimers (residues 4–19, 24–82, 85–97, 101–154 and Mg\(^{2+}\)-\(\alpha,\beta\)-imido-dUTP). Water molecules were included in the model by the water-picking mode of Coot (within hydrogen-bonding distance of hydrogen-bond donor atoms, \(mF_o - DF_c\) density greater than 3.0σ; checked for refined B factors of less than 80 Å\(^2\)).

In this model, the phage dUTPase-specific insert could be nicely located. However, the N-terminal His tag and the C-terminal 15 residues could not be located in the electron-density map, with the sole exception of residue Phe164, which makes a close interaction with the uracil ring of the nucleoside triphosphate ligand. Flexible characteristics of the His tag are a commonly observed phenomenon, while flexible behaviour of the dUTPase C-terminal arm containing the conserved motif V is also frequently observed even in the presence of bound dUTP substrate or its slowly hydrolyzing analogue \(\alpha,\beta\)-imido-dUTP. In addition to protein atoms, the substrate analogue \(\alpha,\beta\)-imido-dUTP was readily observed in the maps, together with a Mg\(^{2+}\) ion coordinating to the phosphate chain of the nucleoside triphosphate; however, the phosphate chain and coordinated Mg\(^{2+}\) ion have higher B factors than the deoxyuridine moiety. An additional Mg\(^{2+}\) ion was also located within the central channel of each trimer.

The final model comprises two dUTPase trimers with \(\alpha,\beta\)-imido-dUTP bound at each active site. A total of 311 water molecules were refined in the final structure. Data-collection statistics and model quality are compiled in Table 1. Structural model figures were prepared using PyMOL (http://www.pymol.org). The coordinates and structure factors have been deposited in the Protein Data Bank under accession code 4gv8.

### 2.3. Mass spectrometry

Samples of \(\varphi 11\) phage dUTPase were analyzed on a Waters Q-Tof Premier-type mass spectrometer in positive electrospray ionization mode. Mass spectra were obtained under native conditions using protein solution containing \(\varphi 11\) phage dUTPase at a concentration of 60 \(\mu\)M in 10 mM aqueous ammonium hydrogen carbonate buffer pH 7.8. This allows transfer of the oligomeric species present in the solution into the gas phase (Benesch et al., 2007; Grandori et al., 2009). The capillary voltage was 2800 V, the sampling-cone voltage was 128 V and the temperature of the source was kept at 363 K. Mass spectra were recorded in the mass range 1500–8000 m/z.

### 2.4. Steady-state kinetics experiments

Proton release during the transformation of dUTP into dUMP and PP\(_i\) was followed at 559 nm at 293 K using a Jasco Acta Cryst. (2013). D69

2.5. Fluorescence measurements

Fluorescence spectra and intensity titrations were recorded on a Jobin Yvon Spex FluoroMax-3 spectrofluorometer in 20 mM HEPES pH 7.5 buffer containing 1 mM MgCl\(_2\), 300 mM NaCl and 10 mM \(\beta\)-mercaptoethanol at 293 K. Tryptophan residues were excited at 295 nm. Emission spectra were recorded between 320 and 400 nm. The excitation and emission slits were 1 and 5 nm, respectively. Additional fluorescence or inner filter effects imposed on the measured intensities during titration experiments were corrected by subtracting the intensity of the assay buffer. Titrations were recorded by using a 5 \(\mu\)M constant protein concentration and varying the \(\alpha,\beta\)-imido-dUTP concentration. Results were fitted to the quadratic binding equation describing 1:1 stoichiometry for the dissociation equilibrium with no cooperativity,

\[ y = x + \frac{A((c + x + K) - ((c + x + K)^2 - 4cx)^{1/2})}{2c}, \]  \(\text{(I)}\)

where \(x\) is the ligand concentration and \(y\) is the fluorescence intensity, \(s = y - x = 0\), \(A\) is the amplitude of the fluorescence intensity change, \(c\) is the enzyme concentration and \(K\) is the dissociation constant (\(K_d\) as before; Takács et al., 2010; Varga et al., 2008; Tóth et al., 2007).

### 2.6. Transient kinetics experiments

Stopped-flow measurements were carried out using an SX-20 (Applied Photophysics, UK) stopped-flow apparatus as described previously (Tóth et al., 2007). Trp fluorescence was excited at 295 nm and emission was selected with a 320 nm longpass filter. Equal volumes (50 \(\mu\)l) of dUTPase enzyme and dUTP solutions in 20 mM HEPES pH 7.5 buffer containing 1 mM MgCl\(_2\), 300 mM NaCl, 10 mM \(\beta\)-mercaptoethanol and 0.1 mM PMSF were mixed. For active-site titration the curves were measured upon mixing various concentrations of dUTP with 20 \(\mu\)M enzyme (post-mixing concentration). 4–5 traces were recorded for each data point. The detector sensitivity was set to high voltage = 357 during measurement. For dUTP binding the curves were measured upon mixing various concentrations of dUTP with 0.5 \(\mu\)M enzyme (post-mixing concentration) and 7–8 traces were recorded. The detector sensitivity was set to high voltage = 460 during measurement. All measurements were performed at 293 K. Time courses were analyzed by Origin 7.5 (OriginLab, Northampton, Massachusetts, USA).

### 2.7. Phylogenetic studies

A total of 61 sequences of \textit{Staphylococcus aureus} phage dUTPases were retrieved from the GenBank database with the homology BLAST search and using GenBank accession numbers extracted from the literature (Kahánková et al., 2010). Global alignments were carried out using ClustalW with automatic penalty preferences. Two distinct superfamilies were identified as corresponding to trimeric and dimeric dUTPases, respectively (Galperin et al., 2006; Moroz et al., 2004, 2005; Vértessy & Tóth, 2009). The groups were confirmed with
3. Results and discussion

3.1. Oligomerization and three-dimensional structure of φ11 phage dUTPase

Fig. 1 shows an alignment of selected dUTPases representing major evolutionary groups of viruses as well as prokaryotes and eukaryotes. The specific insert segment of over 20 residues present in φ11 phage dUTPase as well as in other staphylococcal phage dUTPases (Leveles et al., 2011; Tormo-Másegómez et al., 2010, 2013) is situated between the dUTPase conserved motifs III and IV. Disregarding this insert, the φ11 phage dUTPase sequence corresponds to the usual well conserved sequence characteristics of dUTPases with regard to both the presence of the conserved motifs I–V and the spacing between these motifs (Vértessy & Tóth, 2009).

To investigate the three-dimensional structure of this protein, we used X-ray crystallography on protein crystals grown in the presence of α,β-imido-dUTP, a well characterized isosteric and slowly hydrolysable substrate analogue, as well as Mg²⁺ ion, a cofactor that accommodates the nucleoside triphosphate substrate bound in the dUTPase active site (Kovári et al., 2008; Barabás et al., 2004; García-Nafria et al., 2010; Chan et al., 2004). The structure was determined to 2.1 Å resolution from single crystals using synchrotron radiation, also allowing insights into details of side-chain and ligand conformations.

The structural model built based on the crystallographic data is shown in Fig. 2. The structure shows the characteristic trimeric

Figure 2
Three-dimensional structural characteristics of φ11 phage dUTPase. (a) Overall three-dimensional structure of the protein as a ribbon model of colour-coded subunits (salmon, blue and green) with ligand molecules shown as ball-and-stick models (yellow C atoms and atomic colouring otherwise). The 80k phage dUTPase structure (PDB entry 3zez; Tormo-Másegómez et al., 2013; ribbon model in yellow) is superimposed in order to highlight the position of the phage-specific insert. (b) Ribbon model of a subunit with the phage-specific insert highlighted in purple, superimposed on the respective subunit of 80k phage dUTPase (PDB entry 3zez). The four β-strands of the insert mini-domain are annotated in Fig. 1. (c) Active-site close-up with the catalytically important residues and the Mg²⁺–α,β-imido-dUTP ligand shown as ball-and-stick models, while the rest of the protein is presented as a ribbon model. Mg is shown in green, ligand C atoms in yellow, protein C atoms according to subunit colours and other atoms with atomic colouring. Hydrogen-bonding interactions are shown as dashed black lines.
The phage-specific insert is clearly observed in the density maps as a segment that forms a characteristic β-pleated arrangement looping out from the surface of the trimer (see Fig. 2). The insert consists of four short β-strands (β1–β4; Fig. 1), arranged in pairs of two, constituting an antiparallel organization of a distorted Greek key. The β2 strand of this β-pleated ‘mini-domain’ also contacts a short β-strand of the conserved dUTPase core fold (Fig. 2b). This connection presumably helps to orient the phage-specific mini-domain.

The Mg\(^{2+}\)–α,β-imido-dUTP ligand is accommodated within the active site (Fig. 2c) by making a set of contacts to the conserved side chains of the different motifs in a manner highly similar to those found in human and mycobacterial dUTPases (Chan et al., 2004; Varga et al., 2007, 2008; Mol et al., 1996). The active-site Asp81 coordinates a water molecule posed for in-line attack at the α-P atom of α,β-imido-dUTP. The residues of the C-terminal arm of the enzyme, which contains the conserved motif V, are mostly missing in the electron-density map; however, one side chain (Phe164) could be built in the map. A flexible character of motif V is a common observation in numerous trimeric dUTPases, preventing its localization even in the presence of the nucleoside triphosphate ligand in many cases (Barabás et al., 2004, 2006; García-Nafria et al., 2010, 2011; Samal et al., 2007; Németh-Pongrácz et al., 2007). The fact that the Phe164 side chain is still observable is most possibly owing to the fact that it creates an aromatic stacking interaction with the uracil ring of the ligand (Pecsi et al., 2010; Mol et al., 1996) and hence its conformation is stabilized.
At the N-terminal segment of the mini-domain insert, the three Asp95 residues (shown in red in the alignment in Fig. 1), one from each subunit, participate in coordinating an additional Mg2+ ion per trimer. The electron density for the metal ion and its coordinating atoms are clearly localized within the threefold central channel of the dUTPase trimer (Figs. 3a and 3b). In addition to the three Asp95 carboxyl groups, three water molecules contribute to the hexagonal coordination sphere around the metal ion, in agreement with the geometry usually reported for Mg sites. It is of interest to mention that in the recently published 80k phage dUTPase structure an Ni2+ ion was tentatively localized at this site (with 0.33 occupancy and a high B factor; Tormo-Más et al., 2013).

Localization of an Mg2+ ion within the threefold central channel of trimeric dUTPases has already been reported in enzymes from several sources (Prasad et al., 2000; Takács et al., 2009; Mol et al., 1996; García-Nafria et al., 2010). It is also known that, for example, in human dUTPases the Mg2+ ion within the central channel may contribute to structural stability (Kovári et al., 2004; Varga et al., 2007). In Fig. 1, we aligned all of the dUTPase sequences in which such divalent metal-ion sites have been reported and indicated those glutamate or aspartate residues that were implicated in coordinating the Mg2+ ion in the respective three-dimensional structures. We constructed a structural alignment and present a superimposed structural view of the respective location of these Mg2+ sites in the different enzymes (Fig. 3c). In the presently investigated φ11 phage dUTPase, the Asp95 residue that coordinates the metal ion is the first residue of the phage-specific insert, hence this site is specific to the phage dUTPase. We also found that this residue is present in all staphylococcal phage dUTPases that possess the characteristic dUTPase motifs I-V (Supplementary Figure S1).

Interestingly, this metal-ion site is located at the beginning of the outreaching phage-specific mini-domain insertion. We propose that metal binding may stabilize the conformation of the insertions and may therefore play a role in insert-specific function. Accordingly, it has been reported that a staphylococcal phage dUTPase carrying a glutamate mutation at this position failed to induce the pathogenicity island (Tormo-Más et al., 2010). Our present three-dimensional structure suggests a plausible explanation for this finding: a glutamate at this position cannot produce the same geometry since it

Figure 4
Active-site tryptophan labelling allows the detection of substrate and product binding to φ11 phage dUTPase. (a) The sites of the three aromatic residues serving as fluorophores in the present study, (b), (c) and (d) show the fluorescence spectra of the φ11DUTWT, φ11DUTF108W and φ11DUTF164W enzymes, respectively, in the absence of ligands or in the presence of 1 mM dUMP, 100 μM α,β-imido-dUTP or 500 μM dUTP (saturating concentrations). (e) Fluorescence intensity titration upon α,β-imido-dUTP binding to the φ11DUTF164W construct. Data were fitted using a quadratic equation (see §2) to yield $K_d = 0.32$ μM. Error bars show standard deviations ($n = 3$).
possesses an additional –CH₂– moiety compared with the aspartate and would therefore be sterically incompatible at this site.

Elimination of the phage-specific insert from the phage φ11 dUTPase was stated to result in a catalytically inactive mutant (Tormo-Más et al., 2010). Our presently determined three-dimensional structure in the crystal phase visualizes this insert region as a β-pleated mini-domain within the vicinity of the active site but without any detectable interaction with the active site and with no detectable major interference with the folding of the dUTPase trimer. Since static crystal structures inherently do not provide information concerning flexible movements of protein segments, it was important to design solution-phase experiments.

Tryptophan fluorescence is a frequently used sensitive indicator of protein conformation and conformational changes that occur upon ligand binding. Previously, it has been shown that exchange of the conserved aromatic residue (Phe or His) within motif V to Trp can adequately report on the different signal quenching upon ligand binding of either ligand-bound states during the dUTPase reaction cycle (Pécsei et al., 2011; Varga et al., 2007; Töth et al., 2007). It has been widely reported that motif V undergoes a shift from a flexible to a more ordered conformation during the dUTPase catalytic cycle as it flips over the substrate to contribute to formation of the catalytically competent complex (Vertessy et al., 1998; Vertessy, 1997; Mol et al., 1996; Chan et al., 2004). The characteristic changes in the motif V Trp sensor fluorescence can be quantitatively followed during binding of the substrate analogue α,β-imido-dUTP or the product dUMP. It was also shown that a Trp residue that does not interact directly with the substrate but is in the vicinity of motif V similarly shows characteristic changes in its fluorescence parameters during dUTP hydrolysis catalyzed by dUTPase (Lopata et al., submitted), most probably by sensing the conformational change of motif V. Based on these observations, we created the F108W mutation (φ11dUTP<sup>F108W</sup>) to introduce a Trp residue into the phage-specific insert (Figs. 1 and 4a).

The steady-state activity of this mutant did not change compared with the wild type (φ11dUTP<sup>WT</sup>), as shown in Table 2. We performed fluorescence measurements to determine whether binding of the substrate analogue α,β-imido-dUTP or the product dUMP induces any fluorescence spectral changes in the different Trp mutants. Wild-type φ11 phage dUTPase also contains a Trp between motifs IV and V (Trp143; Figs. 1 and 4a). We first tested whether the fluorescence of this Trp143 changes upon substrate or product binding. This tryptophan residue is not part of the active site and does not contact motif V; therefore it may not sense ligand binding and can be used as a negative control. As expected, none of the ligands induced a significant intensity change or shift in the λ<sub>max</sub> upon ligand binding (Fig. 4b, Supplementary Table S2). Next we tested the φ11dUTP<sup>F108W</sup> mutant that contains a tryptophan fluorophore also within the phage-specific insert and found that this protein also did not show any fluorescence spectral changes upon binding of either nucleotide ligands (Fig. 4c, Supplementary Table S2), indicating that the insert may not contact motif V during when this segment flips over the active site or their interaction may not change upon ligand binding. Although tryptophan fluorescence is a very useful and sensitive measure of protein conformation, it is still an indirect technique in which a lack of signal is indicative of a lack of conformational change but does not constitute direct solid proof.

To complement the fluorescence measurements with a more direct approach, we wondered whether removal of the insert would have any influence on the catalytic properties of φ11 phage dUTPase. Earlier studies concluded that removal of the insert is deleterious to enzymatic activity (Tormo-Más et al., 2010); however, in this case the truncated construct might have been also compromised in folding. Structural knowledge of this protein enabled us to design a truncated construct in which folding may not be perturbed (construct φ11dUTP<sup>A101–122</sup>); in this construct, the first six residues of the insert are still present to potentially serve as a linker segment. We tested the enzymatic activity of this protein and found that both the catalytic rate constant (k<sub>cat</sub>) and the K<sub>m</sub> are only slightly changed compared with the wild-type enzyme (Table 2). We therefore conclude, in contrast to the previous report (Tormo-Más et al., 2010), that the phage-specific insert does not have any significant effect on the catalytic rate constant of dUTPase and its removal does not interfere with the folding of the protein.

### Table 2: Catalytic rate constants (k<sub>cat</sub>) determined for wild-type and mutant φ11 dUTPase proteins.

<table>
<thead>
<tr>
<th>Enzyme construct</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
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<td>1.2 ± 0.5</td>
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<td>φ11dUTP&lt;sup&gt;Fl90W&lt;/sup&gt;&lt;sub&gt;error&lt;/sub&gt;</td>
<td>&lt;10&lt;sup&gt;-3&lt;/sup&gt;</td>
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3.3. Catalytic mechanism of φ11 phage dUTPase

In order to analyze the kinetics of the elementary steps of the dUTPase enzymatic reaction for the φ11 phage enzyme and to determine whether this specific protein is similar or different from other dUTPases (human or bacterial), we exchanged the phenylalanine residue within motif V to tryptophan (yielding the φ11dUTP<sup>F164W</sup> protein), similarly to the previous cases in which this fluorophore was successfully used to analyze the catalytic cycle (Pécsei et al., 2011; Merényi et al., 2011; Töth et al., 2007).

This active-site mutant φ11dUTP<sup>F164W</sup> had the same catalytic rate constant and K<sub>m</sub> value as the wild type (Table 2) and showed very similar signal changes upon ligand binding compared with other dUTPases (Fig. 4d, Supplementary Table S2). The signal quenching upon ligand binding is somewhat less extensive compared with the cases of human or Mycobacterium tuberculosis dUTPase (Supplementary Table S2), presumably owing to the presence of the second tryptophan...
Residue (Trp143) in p11 phase dUTPase that does not change its spectral parameters (Fig. 4b) and therefore ‘dilutes’ the signal. To confirm that the fluorescence change is specific to the ligand binding, we also performed titration with the substrate analogue αβ-imido-dUTP (Fig. 4c). The titration yielded $K_d = 0.32 \mu M$ for the p11 phase dUTPase-αβ-imido-dUTP complex, which is in good agreement with previous data ranging from 0.5 to 2 μM for other dUTPases (Pécsi et al., 2011; Takács et al., 2010; Varga et al., 2007; Töth et al., 2007).

As we obtained a characteristic fluorescence change upon the binding of substrate and product to φ11DUTP$^{164W}$, we also performed fast kinetic active-site titration by stopped flow to observe the intrinsic catalytic constant and real active-site concentration (Fig. 5a). Active-site titration of φ11DUTP$^{164W}$ shows similarly progressive fluorescent curves to those reported for human and M. tuberculosis dUTPases (Pécsi et al., 2010; Töth et al., 2007). The close similarity of the reaction curves that can be fitted with three exponential phases implies that the kinetic mechanism of φ11DUTP$^{164W}$ is also similar to those of the above-mentioned dUTPases (Pécsi et al., 2010; Töth et al., 2007). Accordingly, the main reaction steps are (i) fast substrate binding, (ii) relatively slow substrate-induced isomerization to the rate-limiting hydrolysis step (iii) and (iv) rapid release of the products. The results show that $k_{\text{hydrolysis}} (k_d)$ is 7.6 ± 0.3 s$^{-1}$ for φ11DUTP$^{164W}$.

Based on the titration, the major part (<75%) of the protein preparation is active, indicating that our constructs are fully competent for detailed analysis of structure and function. The measured catalytic rate constant and the measured active proportion of the enzyme preparation are in good agreement with the steady-state activity (Table 2).

Transient kinetic measurement of dUTP binding under pseudo-first-order conditions (Figs. 5c and 5d) showed fast substrate binding and a submicromolar $K_d$. These results further support that the kinetic mechanism of the reaction is similar to that reported for other dUTPases (for a simplified version of the previously proposed kinetic mechanism, see Fig. 5e). In summary, our ligand-binding, steady-state and transient kinetic studies showed that irrespective of the presence of the species-specific insert, the p11 phase dUTPase is also a highly efficient enzyme. Its catalytic cycle

**Figure 5**

Transient kinetics studies describe the elementary steps of the enzymatic reaction. (a) Active-site titration of φ11DUTP$^{164W}$ enzyme by stopped-flow kinetics. φ11DUTP$^{164W}$ enzyme was mixed with equal volumes of varying concentrations of dUTP substrate in the stopped-flow apparatus. The post-mixing concentration of dUTP is indicated on the graph. The smooth line represents triple exponential fits to the single-turnover reaction curves. The reaction curves have two phases with decreasing fluorescence and one with increasing fluorescence. These fluorescence phases were previously determined to be (i) substrate binding, (ii) isomerization and (iii) product release, with the rate determined by the spectrally invisible hydrolysis step (Töth et al., 2007). A triple exponential fit to the reaction curve yielded $k_{\text{observed}} (k_{\text{on}}) = 375 \text{ s}^{-1}$, $k_{\text{on}} = 52 \text{ s}^{-1}$ and $k_0 = 7.9 \text { s}^{-1}$ for 10 μM dUTP and $k_{\text{observed}} = 396 \text{ s}^{-1}$, $k_{\text{on}} = 34 \text { s}^{-1}$ and $k_0 = 7.6 \text { s}^{-1}$ for 15 μM dUTP. (b) shows a representative measurement of dUTP binding. Fluorescence time courses were recorded during the initial binding phase of the reaction at various dUTP concentrations, with a constant dUTPase (φ11DUTP$^{164W}$) concentration of 0.5 μM (post-mixing concentration). Smooth lines are single exponential fits to the experimental curves. (c) and (d) show the analysis of three independent measurements presented in (a). In (c) black scatter data points show the observed rate constants from the exponential fits. Error bars represent the SD for $n = 3$. The smooth line is a linear fit to the data, yielding rate constants $k_{\text{on}} = 21.4 ± 0.7 \mu M^{-1} \text{s}^{-1}$, $k_0 = 17.7 ± 9.5 \text {s}^{-1}$ and $K_d = 0.8 ± 0.5 \mu M$. These rate constants are of the same order of magnitude as for other dUTPases, although somewhat lower, probably owing to the higher salt concentration used in present experiments. In (d) open black scatter data points show the total amplitude change of the fluorescence signal upon dUTP substrate binding. Fitting of a hyperbolic function to the data yielded $K_d = 0.3 ± 0.03 \mu M$. (e) shows a kinetic model of φ11DUTP$^{164W}$ dUTP hydrolysis.
involves fast substrate binding and product release, with the rate-determining step being the hydrolysis of dUTP. The enzyme is characterized by a $k_{\text{cat}}$ of 7.6 s$^{-1}$ that allows it to quickly remove dUTP from the nucleotide pool.

4. Conclusions

Our data showed that the phage-specific insert segment of ϕ11 phage dUTPase folds into a β-sheet mini-domain resembling a distorted Greek-key motif. This small structural motif is very common in protein-folding cores and has also been used for the design of artificial mini-proteins (Dowd et al., 2002; Zheng et al., 2011; Hatfield et al., 2011). Comparison of the presently available sequences of staphylococcal phages encoding trimeric dUTPases (Supplementary Fig. S2) reveals that the presence of such inserts seems to be general and that subgroups of phages can be distinguished based on the segment characteristics (Supplementary Fig. S3). Although the insert sequences are varied, the fold adopted by the insert is very similar in ϕ11 and ϕ80a phage dUTPases (Fig. 2; Tormo-Más et al., 2013). Within the insert, conserved residues can also be identified; the aspartate responsible for coordinating a Mg$^{2+}$ ion (Asp95 in ϕ11 phage dUTPase) is strictly conserved. The structure of the staphylococcal phage ϕ80a dUTPase, belonging to a distinct group (Supplementary Figs. S2 and S3), was also shown to contain the β-sheet mini-domain, although the low resolution prevented the reliable identification of an intrachannel Mg$^{2+}$ ion (Tormo-Más et al., 2013).

To complement the crystal structure analysis, we have shown using kinetics and ligand-binding studies in solution that the phage-specific insert has no effect on the enzymatic activity and that its conformation may not be sensitive to the changes during the catalytic process. Using different site-specific fluorophores, we determined that the phage dUTPase is also a highly efficient enzyme characterized by fast substrate binding and efficient catalysis. These characteristics are even more important in the context of several staphylococcal strains in which a genomic copy of dUTPase could not be identified (Chua et al., 2010; Holden et al., 2010; Baba et al., 2008). Hence, these strains rely on helper phage dUTPases to provide a nucleotide pool with the correct balance between dUTP and dTTP to help genomic integrity.

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