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Active site closure facilitates juxtaposition of reactant atoms for initiation of catalysis by human dUTPase

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Abstract Human dUTPase, essential for DNA integrity, is an important survival factor for cancer cells. We determined the crystal structure of the enzyme:α,β-imino-dUTP:Mg complex and performed equilibrium binding experiments in solution. Ordering of the C-terminus upon the active site induces close juxtaposition of the incoming nucleophile attacker water oxygen and the α-phosphorus of the substrate, decreasing their distance below the van der Waals limit. Complex interactions of the C-terminus with both substrate and product were observed via a specifically designed tryptophan sensor, suitable for further detailed kinetic and ligand binding studies. Results explain the key functional role of the C-terminus.

Keywords: Initiation of catalysis; Tryptophan sensor; High-throughput screening for ligand binding; Enzyme mechanism; Flexible C-terminus

1. Introduction

The ubiquitous dUTPase enzyme keeps cellular dUTP/dTTP ratios at a low level to prevent uracil incorporation into DNA [1,2]. Lack of the enzyme overloads the capacity of base-excision repair leading to chromosome fragmentation and thymine less cell death. In human cancer cells, the enzyme was proposed to be an important survival factor desensitising tumours against drugs perturbing thymidylate metabolism [3,4]. High levels of the nuclear isoform of human dUTPase correlates with bad prognosis in several tumours [5,6]. Targeting dUTPase was therefore suggested as a promising approach in combination with drugs already in clinical use against thymidylate synthase and dihydrofolate reductase.

The human enzyme, like most dUTPases, is a homotrimer with three active sites constructed from five highly conserved motifs and located at subunit interfaces [7]. The flexible C-terminal segment, essential for activity [8,9], was proposed to close over the active site pocket upon substrate binding [7,10–12]. Due to its flexibility, just a few 3D structures could locate this critical segment and unfortunately, these structures did not represent the catalytically competent conformations of the nucleotide ligands, thereby excluding the possibility of mechanistic interpretations [7,10,13,14]. Lack of a robust expression system for any physiological isoform of the human enzyme impeded further progress and necessitated the present study.

We cloned the nuclear isoform of human dUTPase into a high-yield bacterial expression system. The crystal structure of the enzyme in complex with the non-hydrolysable substrate analogue α,β-iminodUTP:Mg was determined and allowed analysis of the full-length C-terminal segment. Solution studies by limited proteolysis and calorimetry were complemented by a novel construct with a highly sensitive tryptophan sensor within the C-terminus. Results suggest that the C-terminal segment may contribute to initiation of catalysis and, in contrast to the *Escherichia coli* enzyme, forms important interactions with both the substrate and the product. The Trp sensor construct is proposed to be used for high-throughput ligand binding assays necessary for drug optimisation.

2. Materials and methods

2.1. Cloning and mutagenesis

The cDNA encoding the nuclear isoform of human dUTPase (a kind gift from Bob Ladner [15]) was cloned into pET3a by conventional PCR amplification. The resulting DUT-N-pET-3a plasmid was subsequently recloned to obtain the N-terminally His-tagged dUTPase (His-DUT-N) wherein the Phe158Trp mutation was introduced by Quikchange (Stratagene) using adequate primers (cf. Supplementary). Cloning was checked by DNA sequencing on both strands. Protein was expressed and purified as described previously [11] also using the NiNTA protocol (Novagen) for the His-tagged constructs. Protein concentration was measured using the molar extinction coefficient calculated from the amino acid composition for the monomer

$\varepsilon_{280\ nm} = 10430\ M^{-1}\ cm^{-1}$ (or $15930\ M^{-1}\ cm^{-1}$, for Phe158Trp mutant).

Enzyme activity was measured according to [16]. Catalytic rate constants were determined to be $8 \pm 3\ s^{-1}$, with no observable change for wild-type His-tagged and Phe158Trp mutant His-tagged species.

Limited tryptic digestion was carried out at 25 °C, using 28 μM dUTPase concentration and 1:250 (w/w) trypsin:dUTPase ratio in 10 mM sodium-phosphate buffer, pH 7.5, containing 150 mM NaCl, 5 mM $MgCl_2$ and saturating concentrations of ligands, if present.

Differential scanning microcalorimetry was performed using a Microcal VP-DSC between 20 and 80 °C at a scan rate of 1 °C/min on 12 μM dUTPase sample in 20 mM TES, 0.3 M NaCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, pH 7.5 buffer.

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Isothermal titration calorimetry experiments were carried out at 20 °C on a Microcal VP-ITC instrument. 270 μM protein in 20 mM HEPES, 1 mM $MgCl_2$, 150 mM NaCl, pH 7.5 buffer was titrated with 3–16 μl aliquots from a stock solution of 2.2 mM α,β-iminodUTP.

Fluorimetric studies were carried out on a Jobin Yvon Spex Fluoromax-3 spectrofluorimeter at 20 °C, with excitation at 295 nm (slit 2 nm), emission between 300 and 450 nm (slit 5 nm). Phe158Trp mutant dUTPase at 1 μM in 20 mM Tris/HCl, 1 mM $MgCl_2$ and 150 mM NaCl, pH 7.5 buffer was measured and titrated by addition of 1–2 μl aliquots from concentrated stock nucleotide solutions.

Mass spectrometry. Data were collected on a MALDI-TOF mass spectrometer (Reflex III, Bruker, Bremen, Germany) (cf. [9,11] and Supplementary material).

N-terminal microsequencing was carried out using the pulsed-liquid phase sequencer ABI 471A of Applied Biosystems, Inc., USA as in [11].

Crystallization and crystallography. dUTPase (282 μM), 1.25 mM α,β-iminodUTP and 10 mM $MgCl_2$ in 10 mM Tris/HCl, 50 mM NaCl, and 0.1 mM TCEP, pH 7.0 buffer was mixed with reservoir solution containing 0.1 M Na–HEPES buffer, pH 7.5 and 18–20% PEG3350 to generate a rhombohedral crystal. Structure of the dUTPase:α,β-iminodUTP:Mg complex was solved by molecular replacement (MOLREP [17]) using a truncated model of the apodUTPase trimer [7] (PDB ID:1Q5U). Electron density for the C-terminal arm of molecule A was clearly visible already in the initial map. The other two subunits of the trimer showed very weak initial densities for residues 147–164. After building of the C-terminus of subunit A, the corresponding residues for molecule B were visualized, and repeated building and refinement steps provided satisfactory fits of these residues (B147–B164) to the observed density. However, the later residues are characterized with relatively high B-factors in the refined model (29.1–58.2, to be compared with the average temperature factor of 20.2 for the whole structure and with corresponding values of chain A: 11.6–29.1), arguing for substantially higher mobility of this C-terminal arm. The C-terminus of molecule C (C147–C164) was not possible to build, even in the final maps. We have to note, however, that water molecules 200–210 roughly trace this part of the C chain. Phe158 from the C monomer is well visible, suggesting that lack of residues C147–C164 was not due to cleavage or exclusion by crystal contacts, but rather originates in more pronounced flexibility. In the final refined structure, careful comparative investigations of crystal packing interactions did not reveal any significant differences around the three C-terminal regions, reinforcing the conclusion that the asymmetry in C-terminus localization is not due to artefacts but may indicate increased conformational freedom of this segment, cf. also similar results in the previously deposited liganded human dUTPase structure PDB ID:1Q5U. Coordinates and structure factor data have been deposited in the Protein Data Bank with accession code 2HQU. Figures were produced using Pymol [18].

3. Results and discussion

3.1. Three-dimensional structural analysis

The overall protein fold of the present structure (resolution 2.2 Å) agrees with earlier studies: three β-pleated monomers form an intricately organized homotrimer by C-terminal arm swapping (Fig. 1A). Novel features, previously not accessible, also emerge: (i) the full-length C-terminal region is visualized for two monomers and (ii) the structure contains an isosteric substrate analogue (α,β-imino-dUTP) in the catalytically competent *gauche* conformation, as well as Mg^{2+} (Fig. 1B) [13,14]. Mg^{2+} accommodation is provided by one oxygen from each of

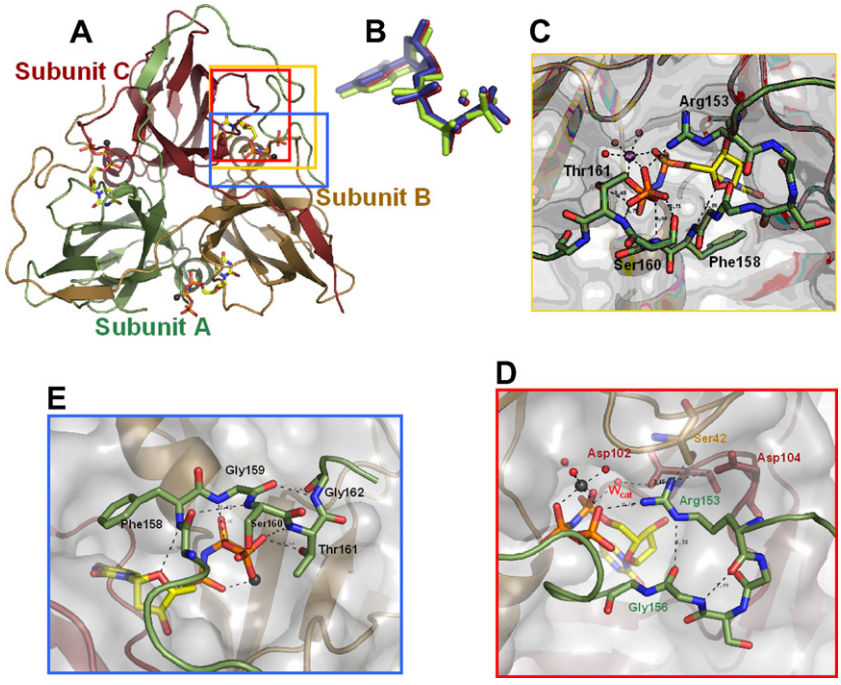

Fig. 1. 3D structure of the human dUTPase:α,β-iminodUTP:Mg complex. Colour-coded ribbon models of the subunits are presented with ligand molecules and Mg-ions shown in ball-and-stick model (yellow carbons and otherwise atomic colouring). (A) Overall 3D structure. Coloured rectangles indicate regions shown in detail on panels C–E. (B) Superimposed ligand molecules as bound in the active sites of the human dUTPase:α,β-iminodUTP:Mg (green), *Escherichia coli* dUTPase:α,β-iminodUTP:Mg (red) and inactive *E. coli* mutant dUTPase:dUTP:Mg (blue) complexes. Note that the phosphate chain conformation of the ligand in the presently determined structure presents the catalytically competent conformation [13]. (C) Interactions of the C-term tail with the ligand molecule. The C-terminal segment of monomer A is shown, with residues and ligand in ball-and-stick model. Ligand carbons are in yellow, protein carbons according to subunit colours, other atoms with atomic colouring. Rest of the protein is presented with ribbon model and transparent surface of the protein core. H-bonding interactions are in dashed lines. Note the extensive number of interactions formed between the C-terminus and the phosphate chain of the ligand that is in contrast to the limited number of interactions formed with the dUMP moiety. For panels (D) Close-up of the interactions formed by Arg153 and (E) β-turns accommodating the phosphate chain of the ligand, graphic representation as in panel C.