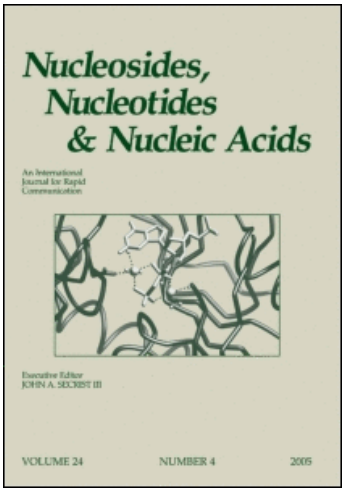

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Mechanistic Studies of dUTPases

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ABSTRACT

The essential enzyme dUTPase is responsible for preventive DNA repair via exclusion of uracil. Lack or inhibition of the enzyme induces thymine-less cell death in cells performing active DNA synthesis, serving therefore as an important chemotherapeutic target. In the present work, employing differential circular dichroism spectroscopy, we show that *D. mel.* dUTPase, a recently described eukaryotic model, has a similar affinity of binding towards α,β-imino-dUTP as compared to the prokaryotic *E. coli* enzyme. However, in contrast to the prokaryotic dUTPase, the nucleotide exerts significant protection against tryptic digestion at a specific tryptic site 20 Å far from the active site in the fly enzyme. This result indicates that binding of the nucleotide in the active site induces an allosteric conformational change within the central threefold channel of the homotrimer exclusively in the eukaryotic enzyme. Nucleotide binding induced allosterism in the *D. mel.* dUTPase, but not in the *E. coli* enzyme, might be associated with the altered hydropathy of subunit interfaces in these two proteins.

Key Words: dUTPase; DNA repair; Anticancer target; Uracil-DNA; Enzyme mechanism; Allosterism.

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INTRODUCTION

Prevention of uracil incorporation into DNA, as provided by dUTPase, is essential for conservation of genetic information.[1] The homotrimer (Fig. 1) catalyzes dUTP hydrolysis into the dTTP precursor dUMP and inorganic pyrophosphate, thereby reducing the dUTP/dTTP ratio with parallel decrease in dUTP and increase in dTTP level in proliferating cells. Lack of dUTPase leads to synthesis of highly uracil substituted DNA that is submitted to excision repair leading to DNA double strand breaks and cell death.[6] The enzyme is highly specific for dUTP (specificity constants in the range of 10^5) in order to prevent wasteful hydrolysis of other high-energy containing nucleotide triphosphates[7,8] (Kovari et al., data to be published). Inhibitors against dUTPase could be efficient agents in antiviral and anticancer therapy.[9] The structural and functional comparison of a pro- (*E. coli*) and a eukaryotic (*Drosophila melanogaster*) dUTPase revealed species-specific differences (Kovari et al., data to be published) which should be taken into account for antagonist design.

Our working hypothesis is that an allosteric conformational change occurs during catalysis in eukaryotic dUTPases, while in prokaryotic enzymes the three active sites are independent (Fig. 1). This hypothesis is based on two experimental findings: 1) different subunit interactions between eu- and prokaryotic dUTPases despite the high structural similarity,[4] 2) the ordered C-terminus in the crystal structure of the human enzyme-dUMP/dUDP complex.[5]

MATERIALS AND METHODS

Recombinant *D. mel.* dUTPase was expressed and purified as in (Kovari et al., data to be published). Circular dichroism (CD) measurements (Kovari et al., data to be published)

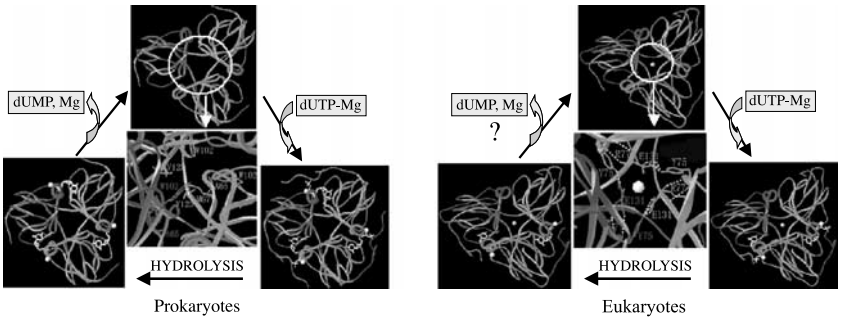

Figure 1. Model for demonstration of the difference in catalytic mechanisms between pro- and eukaryotic dUTPases. In case of the prokaryotic enzyme, dUTP-Mg binding induces C-terminal arm closure. After hydrolysis, the arm pops open to expel the product, in contrast to the eukaryotic dUTPase, where the closed conformer may be retained. Here, partial saturation of the three active sites argues for an eventual allosteric mechanism. Protein subunits are color-coded, ligand is yellow, and Mg^{2+} ion is white. Threefold interfaces are shown in details in the center of both models. The model was constructed using PDB 1EUW,[2] 1F7K,[3] the human and *D. mel.* enzyme structures. (From Refs. [4,5].)

were performed by titrating 43.8 μM fly dUTPase with α,β-imino-dUTP (dUPNPP). Limited tryptic digestion (Kovari et al., data to be published) was realised in the absence and presence of nucleotides (80 μM dUPNPP, 1 mM dUDP or 1 mM dUMP).

RESULTS AND DISCUSSION

Near UV CD spectra of fly dUTPase, dUPNPP and their mixture are presented in Fig. 2A. Addition of dUPNPP to the enzyme significantly enhances positive ellipticity indicating complex formation. The difference signal is a positive peak at 274.4 nm, while the *E. coli* dUTPase-dUPNPP complex is characterised by a negative difference peak at 260 nm.[10] The conclusion is that dUPNPP binding causes different spectral changes in the prototypes of pro- and eukaryotic dUTPases despite the high structural similarity in the active sites.

The strength of the nucleotide binding correlates with the length of the phosphate chain of the nucleotide in both cases[10] (Kovari et al., data to be published). Binding affinities of *D. mel.* dUTPase towards both dUDP and dUMP are, however, much greater than those of the *E. coli* dUTPase[10,11] (Kovari et al., data to be published). The triphosphate dUPNPP shows similar binding to the different types of enzymes [$K_{d,D.\ mel}$ = 0.64 mM (Fig. 2); $K_{d,E.\ coli}$ = 1 ± 0.4 mM, cf.[10] (Kovari et al., data to be published)].

To analyze possible conformational changes responsible for the altered spectroscopic signals (positive vs. negative differential peaks) in the enzyme–dUPNPP complex, limited tryptic digestion, previously shown to be useful in this system,[10,12] was employed. In addition to protection at the tryptic site just preceding conserved Motif 5, and indicative of the closed enzyme conformer (Fig. 3A), binding of dUPNPP to *D. mel.* dUTPase also exerted significant protection at an additional cleavage site. This latter site was identified by mass spectrometric analysis of tryptic digests as the

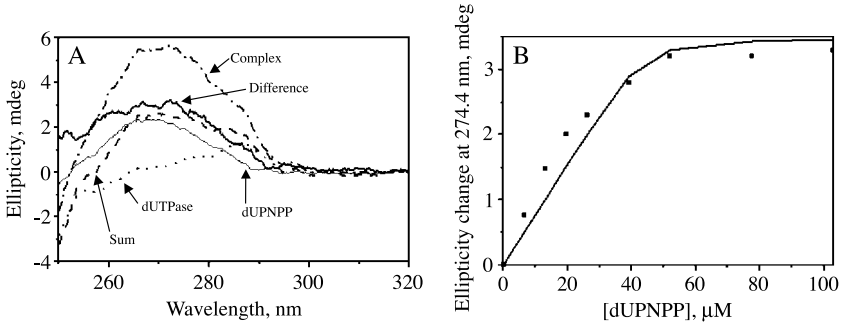

Figure 2. Binding of dUPNPP to *D. mel.* dUTPase (43.8 μM) followed by near UV CD spectroscopy. A: Comparison of spectra recorded and calculated in the dUTPase-dUPNPP (40 μM) system. B: Titration of *D. mel.* dUTPase with dUPNPP. Data points were fitted assuming that the active sites within the trimer are of equal affinity and independent. Note that the fit does not describe faithfully the initial curvature of the titration curve, however, the relatively small number of data points prevented the use of more complex model equations with an increased number of parameters.

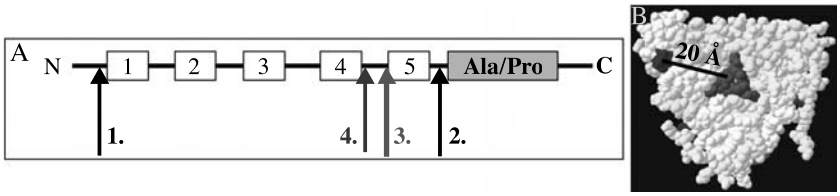

Figure 3. Cleavage sites, in order of decreasing sensitivity, in fly dUTPase during limited tryptic digestion. In addition to boxed conserved dUTPase motifs (A), the fly enzyme contains a C-terminal Ala/Pro rich segment (blue box). Sites #3 (bright blue), at the beginning of Motif 5 in the active site, and #4 (bright red), within the central channel, are also mapped on the surface of the homotrimer (pastel subunits) (B).

Arg132-Ile133 peptide bond that is situated 20 Å away from the nucleotide-binding pocket (Fig. 3B). This result demonstrates that nucleotide-binding induced conformational changes are coupled between the active site and the inner threefold interaction surface of the homotrimer. The closed conformer of the fly enzyme was shown to be formed also in the presence of either dUMP or dUDP (Kovari et al., data to be published), and in these complexes, we found similar protection at the Arg132-Ile133 peptide bond, strengthening the suggestion of the coupled conformational changes. In contrast to the above discussed results, the only tryptic cleavage site amenable to limited digestion experiments in *E. coli* dUTPase is the one preceding Motif 5.[10] Protection at this site in the bacterial enzyme is exerted only in the dUPNPP-dUTPase complex, and not in either enzyme–dUMP or –dUDP complexes, suggesting that the closed conformer requires the full length phosphate chain as present in the intact substrate.

In conclusion, nucleotide binding induced conformational change at a site 20 Å away from the ligand-binding pocket suggests allosterism only in the *D. mel.* enzyme. The suggested allosterism might also be manifested in cooperativity of binding of the nucleotide triphosphate substrate. No such direct experimental observations have yet been reported, however, studies from our laboratory indicate that substrate saturation curves of *D. mel.* dUTPase with dUTP cannot be fitted with the model equation assuming independent active sites (Dubrovay et al., data to be published). Other independent biophysical techniques (e.g., spectroscopy to follow the conformational changes induced by nucleotide binding) may also provide relevant observations on the proposed allosteric mechanism. Results obtained in our laboratory by differential scanning microcalorimetry, mulitdimensional NMR, as well as far-UV CD spectroscopy (Kovari et al. and Dubrovay et al., data to be published) are also in agreement with the working hypothesis and indicate that antagonist design against dUTPases should take into account species-specific differences.

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