Multidimensional NMR Identifies the Conformational Shift Essential for Catalytic Competence in the 60-kDa Drosophila melanogaster dUTPase Trimer*

Zsófia Dubrovay§, Zoltán Gáspári§, Éva Hunyadi-Gulyás§, Katalin F. Medzihradszky‡§, András Pérezcell†, and Beáta G. Vértessy‡ ‡‡

From the Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, POB 7, H-1518, Budapest, Hungary, the Department of Organic Chemistry, Eötvös Loránd University, POB 32, H-1518, Budapest, Hungary, the Mass Spectrometry Facility, Biological Research Center, Hungarian Academy of Sciences, POB 521, H-6701 Szeged, Hungary, and the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446

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The catalytic mechanism of dUTP pyrophosphatase (dUTPase), responsible for the prevention of uracil incorporation into DNA, involves ordering of the flexible C terminus of the enzyme. This conformational shift is investigated by multidimensional NMR on the Drosophila enzyme. Flexible segments of the homotrimer give rise to sharp resonances in the $^1$H,$^{15}$N heteronuclear single-quantum coherence (HSQC) spectra, which are clearly distinguishable from the background resonances of the well folded protein globule. Binding of the product dUMP or the analogues dUDP and $\alpha$-β-imino-dUTP to the enzyme induces a conformational change reflected in the disappearance of eight sharp resonances. This phenomenon is interpreted as nucleotide binding-induced ordering of some residues upon the folded protein globule. Three-dimensional $^{15}$N-edited $^1$H,$^{15}$N HSQC total correlation spectroscopy (TOCSY) and $^1$H-$^{15}$N HSQC nuclear Overhauser effect spectroscopy measurements allowed clear assignment of these eight specific resonance peaks. The residues identified correspond to the conserved C-terminal sequence motif, indicating that (i) this conformational shift is amenable to NMR studies in solution even in the large trimeric molecule and (ii) formation of the closed enzyme conformer in the case of the Drosophila enzyme does not require the complete triphosphate chain of the substrate. NMR titration of the enzyme with the nucleotide ligands as well as kinetic data indicated significant deviation from the model of independent active sites within the homotrimer. The results suggest allosterism in the eukaryotic dUTPase.

The vital role of dUTPases in preventing uracil incorporation into DNA by strict control of cellular dUTP/dTTP levels

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§ Both authors equally contributed to this work.

To whom correspondence should be addressed. E-mail: vertessy@emz.hu.

The abbreviations used are: dUTPase, dUTP pyrophosphatase; HSQC, heteronuclear single-quantum coherence; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

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The vital role of dUTPases in preventing uracil incorporation into DNA by strict control of cellular dUTP/dTTP levels prompts mechanistic investigations on these enzymes. Emerging data indicate that homotrimeric eukaryotic, bacterial, and retroviral dUTPases can be classified into two subfamilies based on the hydrophathy of subunit interfaces and the schedule of conformational changes along the catalytic cycle (1, 2). In particular, a conformational switch of the flexible C terminus containing dUTPase conserved motif 5 seems to be induced in an altered fashion between Escherichia coli and human or Drosophila melanogaster dUTPases (1, 3). In the previous study, experimental evidence has been presented arguing for an increased ordering of the fruit fly enzyme conformation that is induced by both the product dUMP and the nonhydrolyzable γ-phosphate lacking substrate analogue dUDP. Limited proteolysis experiments suggested that a conformational switch within the C-terminal segment contributes to the observed ordering. The experimental techniques, however, allowed only a low resolution structural estimation of the conformational changes. The conformational switch of the C terminus is essential in dUTPase function because it contributes to the formation of the catalytically competent closed enzyme molecular species (4–6). A central question in the catalytic mechanism of dUTPase is whether this closed conformer may be induced only by binding of the γ-phosphate containing substrate dUTP, as in the case of E. coli enzyme, or whether it may be retained in the product-enzyme complex. A high resolution experimental technique was sought that can provide adequate localization of the nucleotide-induced structural changes. It was also desirable to avoid crystallization-induced potential artifacts that may be especially misleading in the investigation of flexible loops capable of adopting an ordered conformation because of crystal packing interactions. The experiments were therefore restricted to the solution phase, and multidimensional NMR spectroscopy was considered to be the method of choice.

High resolution structural analysis in the solution phase still presents a considerable challenge because of the presently observed size limitations of multidimensional NMR spectroscopy. We therefore anticipated that complete structural analysis of the 60-kDa Drosophila dUTPase trimer by NMR spectroscopy is not yet accessible. However, investigations restricted to the flexible regions of the protein were considered to be potentially rewarding. The flexible segments are expected to provide considerably sharper resonance peaks as compared with the resonances associated with atoms in the main bulk globule of the
homotrimer. A similar approach was presented for investigations on E. coli and retroviral dUTPases (7). This study showed that sharp resonances observed in the ligand-free enzyme spectrum disappear upon formation of the complex between dUTPase and α,β-imino-dUTP but do not change when dUDP binds to the enzyme. However, it failed to provide assignment to the observed resonances.

The major aim of the present study was to localize and assign the enzyme conformational changes induced by binding of dUMP, dUDP, or α,β-imino-dUDP in a eukaryotic dUTPase. The Drosophila enzyme was considered to be an excellent experimental object for this attempt because it was shown to contain three segments with distinct degrees of increased flexibilities (3). These segments are as follows: residues 1–15, the 15-residue Drosophila-specific N-terminal region; residues 160–187, the 28-residue Drosophila-specific C-terminal region; and residues 149–159, the 11-residue segment just preceding the Drosophila-specific extension; this last segment contains conserved motif 5, characteristic for all dUTPases throughout evolution (cf. Figs. 1 and 5 in Ref. 3). HSQC, TOCSY, and NOESY spectra of 15N-labeled fly dUTPase allowed clear distinction of NMR resonance peaks and provided complete assignment for residues of dUTPase conserved motif 5. Retention of these segments during the lengthy NMR experiments was controlled by mass spectroscopic measurements. Kinetic analyses and NMR titration experiments suggested cooperativity in saturation of the three active sites.

EXPERIMENTAL PROCEDURES

Chemicals, Enzyme Preparation, and Purification—dUDP and α,β-imino-dUDP were obtained from Jena Bioscience. α,β-Imino-dUTP was prepared by enzymatic phosphorylation of α,β-imino-dUDP (8). The other chemicals were of pro analyti quality from either Merck or Sigma. Recombinant Drosophila dUTPase constructs 1–187 and 1–159 labeled with 15N were expressed in BL21 E. coli cells as described in the previous study (3) grown either on M9 minimal medium containing 15NH4Cl (National Institute of Research & Development for Isotopic and Molecular Technologies, Cluj-Napoca, Romania) or on fully labeled complete 15N medium (Silantes GmbH, Munich, Germany). The supernatant of lysed cells was purified by use of ion exchange chromatogra-

![Image](https://via.placeholder.com/150.png?text=Fig. 1. 1H-15N HSQC spectra of D. melanogaster dUTPase alone (A) and with α,β-imino-dUTP (B), dUDP (C), and dUMP (D). Lines indicate peaks that diminish in intensity and disappear upon enzyme-ligand complexation.)
NMR Properties of Flexible dUTPase Segments

FIG. 2. Intensity changes of $^1$H-$^{15}$N HSQC peaks upon titration of dUTPase with α,β-imino-dUTP (solid symbols), dUDP (open symbols), and dUMP (X-centered symbols). Titration curves are presented for the resonance peak of Ser$^{393}$ (square) that represent residues from motif 5 and the resonance peak of a Gly (circle) from the D. melanogaster-speciﬁc 28-amino acid C-terminal segment.

Enzymatic dUTP hydrolysis was followed spectrophotometrically. Initial velocity data are plotted versus dUTP concentration. The data represent the averages of 5–10 parallel measurements with a standard error of 22%. The data points were fitted to the Hill equation. Parameters of the best ﬁt curve are as follows: turnover number, 12 s$^{-1}$; substrate concentration at half-saturation, 1.2 μM; and Hill coefﬁcient, 2.8.

RESULTS AND DISCUSSION

NMR Assignment of Nucleotide Binding Affected Motif 5 Residues in Drosophila dUTPase—In the $^1$H-$^{15}$N HSQC spectra of full-length (1–187) D. melanogaster dUTPase, ~50 sharp resonance lines could be identiﬁed (Fig. 1A). In addition, a wide background is also observable, with a high number of broad complex resonances. Under our experimental conditions with a 500-MHz frequency, it is expected that resonances that correspond to the main chain and side chain atoms well folded within the 60-kDa homotrimeric dUTPase will be observed only in a broad background (10). Poor dispersion of many Hs and side chain resonances is also apparent from three-dimensional $^1$H-$^{15}$N HSQC TOCSY experiments (not shown). The existence of clear sharp resonances indicates, however, that some moieties of the protein are characterized with an increased conformational freedom. The number of sharp resonances roughly corresponds to the number of residues in the ﬂexible N- and C-terminal segments of the dUTPase chains (cf. protein amino acid sequence in Fig. 1 of the previous study (3)). In the $^1$H-$^{15}$N HSQC spectrum of the 1–159 Drosophila dUTPase construct lacking the C-terminal 28 residues (not shown), the number of observable sharp resonances is only 22, i.e., 28 resonances are missing as compared with the spectrum of the full-length construct. These results, together with the limited proteolysis-based identiﬁcation of the N- and C-terminal ﬂexible segments (3), reinforce the interpretation that the sharp resonances originate from those segments that possess enhanced conformational freedom relative to the rest of the molecule. The retention of these ﬂexible segments during the lengthy NMR experiments was conﬁrmed by mass spectroscopic analysis of the protein before and after NMR spectroscopy (data not shown). No signiﬁcant loss of protein segments was detected.

The addition of α,β-imino-dUTP to dUTPase resulted in the disappearance of eight sharp resonance peaks from the HSQC spectrum (Fig. 1, A and B, lines identify affected residues). The analogue α,β-imino-dUTP is used in this study as an isosteric mimic of the true substrate dUTP (11, 12) that is largely resistant to hydrolysis. The same changes were also induced by the addition of either dUDP (a nonhydrolyzable derivative) or dUMP (product of the enzymatic reaction) to the enzyme solution (Fig. 1, C and D). With the information provided by the three-dimensional TOCSY and NOESY spectra, together with the connectivity requirements of the polypeptide chain in the C terminus, an unequivocal assignment of the affected reso-
Fig. 4. Structural model of the nucleotide-induced conformational changes in Drosophila dUTPase. A, overall model of the homotrimer. The homology model of Drosophila dUTPase (2) is shown in the ribbon representation of color-coded subunits. The ligand dUDP is in ball-and-stick model, colored according to atoms (carbon, white; oxygen, red; nitrogen, blue; and phosphorus, orange). The C-terminal conserved phenylalanine side chain from each subunit is shown stacking over the uracil ring. The model does not contain the flexible N- and C-terminal Drosophila-specific extensions. The white rectangle depicts the model segment detailed in B. B, close-up of one active site. Segments of the protein subunits are shown in the same color code as in A. In addition to the peptide backbone, residues of the C-terminal Gly149-Thr156 octapeptide (blue), as well as the conserved tyrosine at the base of the deoxyuridine accommodating β-hairpin (yellow) and a segment from the third subunit participating in phosphate binding (green), are also shown. Residues Ser155 and Thr156, not localized in the human crystal structure (1), were built in the model using SYBYL (22). Amino acids (Gly154, Ser155, and Thr156 from the blue subunit as well as Arg80 and Ser81 from the green subunit) forming contacts with the phosphate groups and the ligand dUDP are colored according to atoms. Hydrogen bonds connecting protein atoms and...
nances was possible. The assigned segment corresponds to residues Gly144-Glu-Ala-Gly-Phe-Gly-Ser-Thr, located right after the strictly conserved arginine (Arg146) in the protein sequence. These residues comprise conserved motif 5 of dUTPs (13).

Other observed sharp peaks, as far as can be assessed from the analysis of the TOCSY spectra, may well correspond to the other residues in the N- and C-terminal segments of the molecule. The number of resonances assignable as glycines agrees well with that expected from the sequence. Other residues cannot be identified unambiguously, mainly because of the high number of alanine and proline residues in the N- and C-terminal segments, but the information obtainable from the spectral data is in good agreement with the sequence. For instance, the number of strips belonging clearly to U-type residues (Glu, Gln, and Lys) corresponds to the expected composition.

The NMR spectroscopy experiments showed that among the flexible N- and C-terminal residues of Drosophila dUTPase, all residues belonging to conserved motif 5 are modulated by binding of dUMP, dUDP, or α,β-imino-dUTP. Complexation of the product or the di- or triphosphate analogues of the substrate dUTP with the Drosophila enzyme induced broadening of the sharp resonance peaks of motif 5 residues into the background of the folded protein globule. This broadening indicates that the conformational freedom of the residues affected is largely decreased because of binding of dUMP or the other nucleotide phosphates and that in the enzyme-nucleotide complex, motif 5 residues are in a folded conformation in the protein structure. Interestingly, the ordered conformation of motif 5 in Drosophila dUTPase is induced in the same fashion no matter whether the dUMP product or the α,β-imino-dUTP substrate analogue binds to the enzyme. This is in contrast to results reported for E. coli and retroviral dUTPases, where only binding of the triphosphate analogue could induce broadening of some NMR spectral peaks (7). In addition to the suggestions based on previous low resolution data (3), the present NMR experiments provide clear and adequate experimental proof for the hypothesis that the closed enzyme conformer is present with high probability in the enzyme-product complex in the case of a eukaryotic dUTPase. In the closed enzyme conformer, crystal structure data indicate that motif 5 acts as a lid over the nucleus of the ligand in the active site. Retention of three residues in the enzyme-product complex, as shown by the present NMR data, prompts for a mechanism that allows product escape from under the lid. Nonindependent behavior of the three active sites in the homotrimer may provide such a mechanism where subunit communications can modulate active site conformation.

Active Site Saturation in Drosophila dUTPase—Titration experiments were carried out to investigate this possibility. Intensity of all eight resonance peaks shown to broaden upon nucleotide binding, as well as intensity of several other resonance peaks, was followed by NMR spectroscopy along the stepwise titration of the enzyme with either dUMP, dUDP, or α,β-imino-dUTP. At the enzyme concentration of 1.75 mM used in these experiments, the apparent dissociation constants of the dUTPase-dUMP, dUTPase-dUDP, and dUTPase-α,β-imino-dUTP complexes (21, 5, and 0.5 μM, respectively; this last estimated based on the Michaelis constant for dUTP) (3) predicted that after each ligand addition step, the amount of noncomplexed ligand would be under 0.5%, and the concentration of complexed enzyme species would be equal to the concentration of the added ligand up to 100% saturation. A linear relationship between the area under the resonance peaks and the concentration of the chemical species responsible for inducing the resonance peak was assumed. During the titration experiments, the intensity of all the eight decreasing peaks diminished to less than 10% at only 50% saturation. A representative titration curve is shown on Fig. 2 for the resonance peak of residue Ser165. This same behavior was observed during stepwise titration with either dUMP, dUDP, or α,β-imino-dUTP for all eight disappearing peaks. The results suggest that in all the three titrations, partial saturation of the active sites already induces the completion of the conformational shift with regard to folding of motif 5 residues upon the protein globule. These results argue for some cooperativity in the homotrimer that is reflected in the NMR signals.

Kinetic experiments provided an independent assessment of the suggested cooperative behavior. The substrate saturation curve of dUTPase with dUTP (Fig. 3) shows significant deviation from the hyperbolic Michaelis-Menten kinetics. Instead, a strong sigmoid character is evident. Experimental data fitted to the Hill equation (14) resulted in estimation of a Hill coefficient of close to 3 that argues for a quite strong cooperative character in the homotrimer.

Taken together, NMR titration experiments and the kinetic data both indicate a strong deviation from the model of independent active sites in the Drosophila dUTPase homotrimer. Such behavior has not yet been reported for any dUTPase. Instead, based on kinetic and nucleotide binding data, the absence of any cooperativity was ascertained in several references (15, 16). In the previous study, ligand binding data as followed by circular dichroism difference signals could also be fitted to the simplest model. The information content of these data seems to be surpassed here by the highly sensitive NMR spectroscopic experiments. The physiological significance of the cooperative behavior observed in the Drosophila dUTPase homotrimer is not yet evaluated. We propose that this character increases the sensitivity of the enzyme toward regulatory mechanisms eventually required in the more developed organisms. Lack of such sensible character in the E. coli enzyme might be due to the fact that in pyrimidine metabolism of enterobacteria, dUTP is an obligate precursor for dTTP biosynthesis (17). In eukaryotes, dUTPase is also available for dTTP biosynthesis as the product of DCP deaminase. This enzyme is not present in enterobacteria that rely entirely on formation of the uracil ring from cytosine at the level of triphosphate nucleotides by dCTP deaminase. It is therefore probable that the increased dUTP load in E. coli requires the presence of a robust enzyme that can efficiently remove dUTP from the DNA polymerase route under varied cellular conditions. Multiple examples indicate that enzyme regulation in eukaryotes is more complex in many cases to enhance fine-tuning of cellular pathways (18–20). The difference in subunit interaction interfaces between prokaryotic and eukaryotic dUTPases may provide a structural reason for the observed absence or presence of cooperative character in E. coli and D. melanogaster enzymes.

In addition to the observed decrease of motif 5-associated spectral peaks, the NMR titration experiments also showed that resonances from several side chains of the Drosophila-specific 28-residue-long C-terminal segment are characterized by a minor but significant increase in intensity induced by the dUDP or α,β-imino-dUTP binding to dUTPase. These resonances, however, do not change during titration of the enzyme.
with dUMP. Fig. 2 plots the data for a representative glycine from this region. These data indicate that binding of substrate to the dUTPase active site may induce conformational perturbations in the Drosophila-specific 28-residue-long C-terminal flexible extension, whereas product binding has no such effect.

**Structural Model for Motif 5 Conformation**—The results from the multidimensional NMR spectroscopy showed that the conformation of motif 5 residues is closely similar in enzyme-dUMP and enzyme-α,β-imino-dUTP complexes. Modelling of these residues was attempted based on crystal structure data for dUTPase-ligand complexes, and the previously reported homology modeling of Drosophila dUTPase structure (1, 2, 21) (Fig. 4A). The last two residues (Ser155 and Thr156) in the peptide segment that are modulated by nucleotide ligand binding to the enzyme could not be localized in the previous human crystal structure. We therefore simulated the possible conformation of these two residues by energy minimization (22). Analysis of the modeled structure revealed that the Gly152-Phe-Gly-Ser-Thr pentapeptide of motif 5 forms multiple interactions with the phosphate chain of α,β-imino-dUTP (Fig. 4B). The hydroxyl side chain groups of both Ser155 and Thr156 form hydrogen bonds to the oxygens of the α-phosphate group of the ligand. These interactions may well be determinant in the ordering of motif 5 even in the dUTPase-dUMP complex, because the α-phosphate oxygens in dUMP may also be expected to form the same hydrogen bond with the Ser155 and Thr156 side chain hydroxyl groups. In addition to side chain interactions, main chain carbonyl oxygen and amide nitrogen atoms also participate in hydrogen bonding to phosphate oxygens. Interactions between residues and main chain atoms of the Gly149–Thr156 octapeptide (e.g. an hydrogen bond network from Ser156 side chain oxygen through Gly152 main chain nitrogen to Gly151 carbonyl oxygen) contribute to the stabilization of the phosphate chain accommodating conformation of the C-terminal segment (Fig. 4B). The Gly149–Gly152 tetrapeptide also forms interactions with the surface of the neighboring protein subunit (not shown) that help in orienting the C-terminal segment toward the ligand phosphate chain.

Based on the experimental results of ligand-induced cooperative conformational changes in Drosophila dUTPase, we propose the following scheme for the catalytic cycle (Fig. 4C). Binding of one molecule of substrate to the homotrimeric apoenzyme induces conformational changes in all the three active sites that will facilitate binding of further molecules of substrate. Cleavage of the scissile bond does not necessarily induce major conformational changes; the uracil, deoxyribose, and α-phosphate moieties of the substrate and the product experience the same binding environment before and after catalysis. The similar cooperative ordering of the C-terminal segment in the enzyme-dUTP or enzyme-dUMP complexes argues that a homotrimer partially saturated with either the substrate dUTP or the product dUMP may be equally primed for the binding of additional substrate molecules. The physiological relevance of this possibility is that within the cell, dUTPase may respond to both dUTP and dUMP concentrations and will be present in the catalytically competent conformation with a higher probability. An interesting hypothesis is put forward in this scheme, namely that product escape or product-substrate exchange in one of the active sites may be coupled to catalytic cleavage in the neighboring active site. The lack of cooperative characteristics and product-induced ordering of the active site in the prokaryotic E. coli dUTPase (5, 7, 15) on one hand and the high similarity of human and Drosophila dUTPases (2) on the other hand suggest that the dUTPase homotrimer has acquired cooperativity during evolution.

**Conclusions**—Multidimensional NMR spectroscopy is shown to be adequate to provide high resolution conformational characterization of flexible segments of a 60-kDa protein in the solution phase. In Drosophila dUTPase, this method reveals that motif 5 residues adopt the same ordered conformation in the enzyme-product complex as in the enzyme-α,β-imino-dUTP complex. The retention of the closed enzyme conformer within the enzyme-product complex is coupled to cooperativity within the eukaryotic dUTPase homotrimer.

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