dUTPase is responsible for preventive DNA repair via exclusion of uracil. Developmental regulation of the Drosophila enzyme is suggested to be involved in thymine-less apoptosis. Here we show that in addition to conserved dUTPase sequence motifs, the gene of Drosophila enzyme codes for a unique Ala-Pro-rich segment. Kinetic and structural analyses of the recombinant protein and a truncation mutant show that the Ala-Pro segment is flexible and has no regulatory role in vitro. The homotrimer enzyme unfolds reversibly as a trimeric entity with a melting temperature of 54 °C, 23 °C lower than Escherichia coli dUTPase. In contrast to the bacterial enzyme, Mg\(^{2+}\) binding modulates conformation of fly dUTPase, as identified by spectroscopy and by increment in melting temperature. A single well folded, but inactive, homotrimeric core domain is generated through three distinct steps of limited trypsinolysis. In fly, but not in bacterial dUTPase, binding of the product dUMP induces protection against proteolysis at the tryptic site reflecting formation of the catalytically competent closed conformer. Crystallographic analysis argues for the presence of a stable monomer of Drosophila dUTPase in crystal phase. The significant differences between prototypes of eukaryotic and prokaryotic dUTPases with respect to conformational flexibility of the active site, substrate specificity, metal ion binding, and oligomerization in the crystal phase are consistent with alteration of the catalytic mechanism and hydropathy of subunit interfaces.

DNA repair processes and an exquisite fine-tuning of nucleotide metabolism is required for genome stability and maintenance. The enzyme dUTPase catalyzes the hydrolysis of dUTP into dUMP and anorganic pyrophosphate using a metal ion (Mg\(^{2+}\)) (1, 2). Through this reaction, the enzyme keeps the cellular dUTP/dTTP ratio at a low level by depleting the dUTP pool and contributing in parallel to dTTP biosynthesis (3). Lack of the enzyme leads to massive uracil incorporation into DNA, repair of which via base excision, an efficient pathway to remove uracil, progresses to a hyperactive futile cycle resulting in DNA double-strand breaks, chromosome fragmentation, and cell death (4, 5). dUTPase is therefore reported to be a preventive DNA repair factor (6), essential in both prokaryotes (7) and eukaryotes (8), and a key determinant of viral host cell range and infectivity of Herpes simplex virus (9), as well as several retroviruses (10–12).

Drugs targeting enzymes of de novo thymidylate biosynthesis, e.g. fluorouracil against thymidylate synthase or methotrexate against dihydrofolate reductase, are widely used in anticancer and antiviral chemotherapy (13). Their molecular mechanism of action is to induce grave perturbances in the cellular dUTP/dTTP pools that result in thymine-less cell death preferentially killing cells with actively ongoing DNA synthesis, e.g. tumor and virus-infected cells (14). dUTPase inhibition would obviously result in similar effects. The importance of thymine-less cell death is underlined by the fact that this apoptotic pathway is independent from p53 signaling, at least in hematopoietic cells (15). Quite recently, activation of dUTPase gene expression in p53 mutant tumor cells argued for a particular significance of dUTPase in tumor development and survival (16). Moreover, cellular studies in human cancer cell lines, which show development of resistance against fluorouracil via dUTPase overexpression (17, 18), indicate that benefits and success of the anticancer therapy based on inducing thymine-less cell death may ultimately depend on a critical interplay among enzymes of thymidylate metabolism. In addition to its importance as a novel chemotherapeutic strategy, dUTPase inhibition is also suggested to play a significant role in developmental apoptosis, at least in Drosophila melanogaster (19). It is therefore of immediate interest to investigate these enzymes. A wealth of data and in-depth analyses are available on thymidylate synthase and dihydrofolate reductase, but our knowledge on dUTPases is lagging behind. For human dUTPase, a series of thorough studies (18, 20–23) addressed cellular level and regulation of physiological isoforms, and a crystal structure was also published (24). However, no enzymological characterization is yet available for any eukaryotic dUTPase.

Eukaryotic, bacterial, and retroviral (EuBaR)\(^1\) dUTPases are

\(^1\) The abbreviations used are: EuBaR, eukaryotic, bacterial, and retroviral; DSC, differential scanning calorimetry; FIV, feline immunodeficiency virus; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino]ethanesulfonic acid; MS, mass spectrometry.
usually homotrimers where each β-pleated subunit forms its own jelly roll topology by swapping the C-terminal β-strand of the neighboring subunit, as shown in the crystal structures (24–27). Despite limited sequence homology, the dUTPase trimeric fold seems to be well conserved from bacteria to man and even in retroviruses. Active site architecture also follows a common pattern where the three active sites of the homotrimer, located in clefts between neighboring subunits, are built in a fashion of basically 3-fold symmetry recruiting conserved motifs from all subunits (24, 27–29). Two successive β-strands and their connecting turn constitute the core of the active site that accommodates the uracil and deoxyribose rings of the substrate dUTP. Phosphate recognition is provided conserved motifs of the neighboring subunit. The third subunit contributes its C-terminal fifth conserved motif, the ordering of which upon the active site induces the catalytically competent closed conformation (24, 29, 30). Despite the overall similarities, the available data suggest the existence of marked differences between prokaryotic and eukaryotic dUTPases. First, structural superpositions and sequence alignments have indicated that the 3-fold inner channel of the trimer is densely packed with hydrophobic side chains, a divalent metal ion (Mg$^{2+}$) has been localized at the base of this inner channel in the crystal structure of the human enzyme (24), 18 Å away from the active site. There is no place for such a metal ion in the structure of the prokaryote Escherichia coli dUTPase where the 3-fold channel is densely packed with hydrophobic side chains. Third, based on crystal structure data of human and E. coli dUTPases, the crucial conformational change leading to the formation of the catalytically competent closed enzyme conformer may occur with an altered timing in these two enzymes. This conformational change is realized by intricate subunit interactions as the C-terminus of one subunit crosses over the neighboring subunit and adopts an ordered conformation to contact the substrate bound in the active site of the third subunit. In the bacterial enzyme, detailed functional investigations (2, 30, 33, 34), in agreement with the crystal structure in complex with the nucleotide dUDP (28), indicate that the γ-phosphate of the substrate is indispensable in inducing the closed conformer, and after catalytic cleavage the active site pops open. In the case of the human enzyme, the crystal structure suggests the retaining of the closed conformer even with the product dUMP (24), but there are no functional data published. This difference in active site conformational rearrangements, dependent on subunit interactions, suggests that the catalytic cycle may be altered in the bacterial and human dUTPases.

A critical assessment of this hypothesis needs functional experiments in the solution phase because crystallization conditions are prone to induce ordering of loops, which are flexible in solution, via crystal packing interactions.

An earlier study reported the presence of dUTPase in D. melanogaster, stating that the enzyme is a monomer with a molecular mass of 45 kDa (35). However, all EuBaR dUTPases characterized to date are homotrimers with a subunit molecular mass of 15–18 kDa. We recently identified a sequence in D. melanogaster expressed sequence tag data bases encoding a putative dUTPase containing all the five conserved motifs (31). To resolve the contradiction and provide a eukaryotic dUTPase for functional investigations, in the present study we report cloning of D. melanogaster dUTPase together with enzymological, structural, and initial crystallographic characterization of the recombinant protein. Details of structural and functional differences of E. coli and D. melanogaster dUTPases indicate alterations in active site flexibility, Mg$^{2+}$ ion binding, substrate specificity, and oligomerization.

EXPERIMENTAL PROCEDURES

Electrophoresis materials and Chelex resin were obtained from Bio-Rad, and other chromatography resins and columns were purchased from Amersham Biosciences. Phenol Red indicator and ammonium bicarbonate were obtained from Merck. Nucleotides, buffer substances, and other materials of analytical grade purity were purchased from Sigma. Restriction enzymes and other molecular biology materials were purchased from Stratagene, unless stated otherwise. E. coli dUTPase was purified as described previously (36).

Plasmids, Vectors, and Bacterial Strains—The plasmid clone LD 20050 (Research Genetics, Inc.) harboring the D. melanogaster dUTPase gene (31) constituted the starting material for cloning. The plasmid pET22b (Novagen) in the E. coli strains BL21(DE3), ER2566, JM109(DE3), and AD494(DE3) was used for protein expression. E. coli XL1-Blue (Promega) was used for plasmid amplification.

Cloning and Expression Vector Construction—The plasmid LD 20050 was used as a template for amplification of the D. melanogaster dUTPase gene by PCR. The PCR was performed with the 5′-ACAAGATCTcaTatgccaAGCaccgatttcgccgacattc and two different primers 5′-ctgAGGACTTGTcactgtgacgttgagagactgtgcagtcgctagctcctgGAGACTTGAAATGACGAGATTGTTCACTTGAGTGCAGTCCTGAGTCTcactgtgacgttgagagactgtgcagtcgctagctcctg and 5′-GAGACTTGAAATGACGAGATTGTTCACTTGAGTGCAGTCCTGAGTCTcactgtgacgttgagagactgtgcagtcgctagctcctg (uppercase denotes nonpairing bases), for generating two different D. melanogaster dUTPase constructs containing residues 1–159 or residues 1–187, respectively (cf. cDNA and translated protein sequence in Ref. 31). Together, these primers introduce an EcoRI (GAATTCT) site just upstream of the gene, an NdeI (CATATG) site fused into the start TAG codon, together with either a single XhoI site (CTCGAG) downstream of the gene (short construct, for producing dUTPase (1–159)), or a combination of an XhoI site and a BglII site (AGATCT) (this latter fused into the stop TAG codon, long construct for producing dUTPase (1–187)). The BglII site was introduced to facilitate distinction between the short and long DNA constructs whose lengths differ only by ~80 base pairs. Additionally, the TCA as codon of the original cDNA was changed into AGC as more frequently used in E. coli. The PCR product, created by using Pfu polymerase, was purified and concentrated using the QiAQuick PCR purification kit and then digested with EcoRI and XhoI. The fragments were run on agarose gels, and the gene of the correct length was excised, purified using a Sephaglass DNA purification kit and ligated overnight into the pET22b vector (previously digested with EcoRI and XhoI and purified with the Sephaglass DNA purification kit) using NEB ligase (New England BioLabs). These pET22 constructs, containing either the shorter or the longer construct of the D. melanogaster dUTPase gene, were digested with NdeI/PstI, and the resulting fragments were ligated into pET22b digested with NdeI/PstI. The resulting recombinant pET22b plasmids, containing the D. melanogaster dUTPase gene, were named SDdut-pET22b and LDdut-pET22b. These plasmids were electrotransformed into E. coli XL1-Blue. The transformed cells were selected on LB medium containing Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and ampicillin, and colonies were grown in liquid culture for 18 h. The plasmid SDdut-pET22b transformed E. coli XL1-Blue, resulting in a low number of recombinant colonies, reflecting some degree of intolerance between the eukaryotic dUTPase gene containing vector and the XL1-Blue strain. Nevertheless, these recombinant colonies could be used further for plasmid production, albeit with low efficiency. The intolerance between plasmid LDdut-pET22b and XL1-Blue was even more evident, excluding the appearance of transformed colonies in this case. The problem was circumvented by pasting together the first half of the short construct (already proven to co-exist in pET22b with D. melanogaster dUTPase (1–187)) in intact form, proving to result in a small number of viable transformant colonies and adopted the previously used determination LDdut-pET22b.

Plasmids LDdut-pET22b and SDdut-pET22b also showed some intolerance in various other E. coli strains (ER2566, JM109(DE3), and AD494(DE3)), resulting in a low efficiency of transformation. Nevertheless, expression was at high level in the transformants. It is worthwhile to note that in our parallel experiments, the D. melanogaster gene cloned into the high copy number pKS plasmid gave a high frequency of recombinants in all the above listed E. coli strains. The fact that it was...
a version of plasmid pKS, containing the T7 promoter region, that could be successfully used for the cloning of the human dUTPase gene (24) may suggest the presence of some common characteristics between these eukaryotic dUTPase genes causing adversity interference with pET vectors in E. coli.

The inserted DNA and flanking regions of plasmids SDdut-pET22b and LdDut-pET22b were sequenced on both strands using ABI PRISM 2.1.0 (Applied Biosystems). Oligonucleotide primer synthesis and automated DNA sequencing were performed using the Biological Research Center facilities in Szeged, Hungary.

**Expression and Purification of Recombinant D. melanogaster dUTPase**—The recombinant plasmid SDdut-pET22b or LdDut-pET22b was transformed via electroporation into E. coli BL21(DES) or E. coli DH5α (pK 3A) or DH5α (pK 3B) containing the plasmid pET22b. Transforms were grown on ampicillin-containing standard LB-agar plates overnight at 37 °C and were used to inoculate 500 ml of LB medium, supplemented with carbenicillin in 2-ml Erlenmeyer flasks.

The cells were propagated at 37 °C on aerated shaker platforms until the culture reached the exponential growth phase as followed by measuring optical density at 600 nm. At this phase (usually at 0.5–0.4 readings), the synthesis of T7 RNA polymerase was induced by the addition of isopropyl-β-thio-galactoside to a final concentration of 0.5 mm, and the cells were grown for an additional 5–6 h. The cells were harvested through centrifugation at 8,000 × g for 10 min and stored at −70 °C.

The cell pellets were solubilized in 1% volume of lysis buffer (50 mM Tris-Cl, pH 8.5, containing 1 mM dithiothreitol (0.5 mM phenylmethylsulfonyl fluoride) on ice. Following the addition of 50 μg/ml lysozyme, 1 μg/ml RNase, and 1 μg/ml DNase, cell suspensions were stirred on ice for 30 min, sonicated (3–5×60 s), stirred again for 20 min on ice, and centrifuged (15,000 × g for 20 min at 4 °C). Final supernatants were loaded on a Q-Sepharose ion exchange column (200 ml) equilibrated in 25 mM sodium phosphate pH 7.5 buffer containing 1 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride and developed using a total volume of 700 ml linear gradient from 0 to 1 M NaCl in the equilibration buffer, as created by the Gradifrac system (Amersham Biosciences). D. melanogaster dUTPase appeared in fractions at 0.5–0.6 M NaCl, as followed by enzyme activity measurements and SDS-PAGE analyses depending on enzyme purity. Gel filtration of dUTPase on Superdex 200 HR column (Amersham Biosciences) was also employed, if necessary. The purified preparations appeared as single bands on SDS-PAGE gels, when investigated by densitometry, suggesting at least 95% purity. Enzyme stocks were concentrated on Millipore centrifugal filters (10-kDa cut-off) to 8–15 mg/ml final concentration, flash frozen in liquid nitrogen, and stored at −70 °C. As shown by enzyme measurements and SDS-PAGE, this storage did not cause considerable degradation up to 3 months. More prolonged storage and several cycles of freeze/thaw did cause slight fragmentation, as observed on SDS-PAGE (cf. Fig. 5A, lane 11), without any effect on the catalytic activity. Throughout the present study, molar enzyme concentrations refer to the stock solutions, unless stated otherwise. Before use, aliquots of the enzyme were dialyzed against respective buffers.

**Protein Concentration**—Protein concentration was measured either by Bradford’s assay (38), using the protein determination kit from Bio-Rad, and bovine serum albumin as standard) or spectrophotometrically using $A_{280}$ nm = 0.02, 0.26, or 0.52, for the D. melanogaster dUTPase constructs 1–159, 1–187, or E. coli dUTPase, respectively, as calculated from amino acid composition or determined experimentally (39).

**Discontinuous Qualitative dUTPase Enzyme Activity Assay by Thin Layer Chromatography**—For fast and convenient check of enzyme activity in crude extracts, where buffer composition presented experimental problems, thin layer chromatography was employed. For-UV CD spectra were recorded on a JASCO V550 spectrophotometer in a 1-mm-pathlength thermostatted cuvette at 25 °C, using the protein samples at 0.2 mg/ml concentration in 20 mM potassium phosphate buffer, pH 7.5, and adding <0.5-μl aliquots of concentrated MgCl₂ stock solution. Three scans of every spectrum were averaged. Spectral data processing was done by using the built-in JASCO software of the spectrophotometer. Far-UV CD spectra were generated by the k2d method for an estimation of secondary structural elements.

**Analytical Gel Filtration**—Analytical gel filtration was conducted on Superdex 200 HR column calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen, and RNase (molecular masses of 67, 43, 25, and 13.7 kDa, respectively). Calibration was performed likewise in the above buffer also containing 1 mM dUDP or dUMP.

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Electrospray Mass Spectrometric Measurements—Electrospray mass spectrometric measurements were carried out on a Applied Biosystems/MD Sciex API 2000 triple quadrupole instrument. A standard sample preparation procedure was applied on the protein sample. Trypsin was used for digestion in aqueous solution using a protein:trypsin ratio of 10:1. The tryptic peptide fragments were then nanosprayed using a home-build nanospray ion source. The most intense peaks were selected for MS/MS sequence analysis experiments. Alternatively, the samples before and after limited trypsinolysis were nanosprayed and measured without further digestion.

Enzymatic Synthesis of dUDP—At the beginning of this work, dUDP became commercially unavailable, necessitating synthetic efforts. Enzymatic phosphorylation of dUMP is a straightforward approach. Nucleoside monophosphate kinases usually require ATP as the phosphate donor and can phosphorylate a variety of nucleotides with the simultaneous production of ADP (44). The relatively low substrate specificity of these enzymes encouraged us to design the synthesis experiment using dUMP as the phosphate acceptor and dUTP as the phosphate donor, resulting in the single product of dUDP (dUMP + dUTP = 2 dUDP). Nucleoside monophosphate kinase was obtained from Roche Applied Science (catalog number 107948) in the form of lyophilized powder. It was dissolved at 25 mg/ml concentration in 150 mM ammonium bicarbonate buffer, pH 8.5, containing 25% (v/v) glycerol. The reaction mixture for dUDP synthesis contained 0.9 mg/ml nucleoside monophosphate kinase, 10 mM dUTP, 20 mM dUMP, 5 mM MgCl2 in 150 mM ammonium bicarbonate buffer, pH 7.2. The reaction reached equilibrium in 72 h under stirring at 4 °C. The nucleotides were separated by ion exchange chromatography (Q-Sepharose or MonoQ column), using 0–600 mM ammonium bicarbonate gradient.

Differential Scanning Calorimetry (DSC)—Calorimetric measurements were made in a high sensitivity differential scanning calorimeter (MicroCal MCS instrument). The experiments were performed between 20 and 90 °C at a scan rate of 1 °C/min. The protein samples dissolved at 0.2 mg/ml concentration in 5 mM Hepes, 0.1 M NaCl, 1 mM dithiothreitol pH 7.5 buffer, as well as the buffer for reference were degassed by stirring under vacuum for 15 min at room temperature immediately before loading into sample and reference cells. The samples and the measuring cells were kept under pressure to prevent any degassing during heating. The base line for each run was determined in an identical experiment with buffer in both cells and was subtracted. The melting temperature (Tm) was defined and taken to be the maximum of the relative heat capacity capacity. Calorimetric enthalpies were calculated as equal to the area under the heat absorption curves; molar calorimetric enthalpies were calculated by taking into account the molar concentration of the protein sample (45). The measured heat capacity functions were fitted with theoretical models assuming simple two-step transitions (molecule population consisting of either folded or unfolded species with no experimentally observable intermediates) and reversibility of the transition. Fitted model coefficients were refined by the van ‘t Hoff equation (46) (35). All of the calculations and model fitting were performed using the built-in Origin software (Microcal) for evaluation of DSC experiments.

Dynamic Light Scattering Measurements—Dynamic light scattering measurements were carried out on a DynaPro-MXI molecular sizing instrument (Protein Solutions) equipped with a 20-μl microsampling cell at either 20 or 4 °C, according to the manufacturer’s recommendations. The sample contained 5.5 or 2.8 mg/ml protein in 50 mM Tris, 50 mM NaCl, and 1 mM dithiothreitol, pH 7.0, or 30% 2-methyl-2,4-pentanediol, 50 mM acetate, 0.2 M NaCl, pH 4.6, respectively. An aliquot of 25 μl was freshly filtered (through a 0.02-μm Whatman Anodisc membrane filter unit) into the measurement cell. 10–100 readings were recorded. All data were analyzed using the non-negatively constrained least squares method (46) incorporated into Dynamics software (version 5.20.05; Protein Solutions). The analysis is based on the Stokes-Einstein equation (Dx = kBT6πηSV/Rp), under the assumption of Brownian motion. Dx (translational diffusion coefficient) was converted to the hydrodynamic radius (Rt of the sample particles (kT is Boltzmann constant, η is the absolute temperature in degrees Kelvin, P is the value of the constant P, and SV is the solvent viscosity). Polydispersity was defined as (standard deviation of Rti/Rm, giving an estimation of the homogeneity of particle sizes in the sample (47).

Crystalization—The protein solutions used for crystallization were dialyzed overnight against a buffer containing 50 mM Tris, 50 mM NaCl, and 1 mM dithiothreitol, pH 7.0. The samples were frozen in liquid nitrogen and stored at −70 °C. The crystallization conditions were screened in various buffer systems at different pH values and temperatures (Crystal Screen, Hampton Research). Vapor diffusion experiments were screened in hanging and sitting drops. The enzyme solutions contained 10 mM Mg2+ ions, and dUDP in 2–5-fold molar excess over the enzyme concentration. The enzyme solution (2 μl) was mixed with an equal volume of the reservoir solution. The drops were equilibrated against 500 μl of reservoir solution. The crystals could be obtained at acidic pH values (pH 4.6) from crystallization conditions containing 20–25% 2-propanol or 25–35% 2-methyl-2,4-pentanediol as precipitant agent and some salt in low concentration (e.g. 0.2 M CaCl2 or NaCl). The appropriate enzyme concentrations were in the range of 3–9 mg/ml. Crystals up to 0.3 mm in length and 0.2 mm in the other dimensions were obtained at 4 °C from 7.2 mg/ml D. melanogaster dUTPase (1–159) solution in presence of 10 mM MgCl2, 2.5 mM dUDP, 0.2 mM CaCl2, and 20% 2-propanol in 0.1 mM sodium acetate buffer, pH 4.6, after a week of incubation. Representative crystals were used for x-ray data collection.

X-ray Data Collection—Synchrotron radiation was used to collect diffraction data at 100 K from a single crystal of dimensions 0.25 × 0.2 × 0.2 mm. The crystal was cryoprotected in reservoir solution supplemented with 15% 2-methyl-2,4-pentanediol and then flash frozen in a nitrogen stream at 100 K. The data were collected on the D832 Beamline at LURE, Orsay. The diffraction patterns were collected with a 345-mm MAR research image plate scanner. The data sets were indexed and integrated using the MOSFLM 6.01 program (48). All subsequent programs used (SCALA (49) and TRUNCATE (50)) were from the CCP4 program suite (Collaborative Computing Project, version 4.0) (51). The statistics for the data collection and processing are listed in Table IV.

Molecular Replacement—Molecular replacement and the calculation of self-rotation functions were carried out using the AMoRe program (52) of the CCP4 4.0 package. The amino acid sequence identity between the human and the short construct D. melanogaster dUTPase 1–159 is 60%. Therefore, the molecular replacement was carried out using the crystal structure of one monomer of the human dUTPase (24) as a search model. The results gave one top hit. The correlation coefficient for this peak was 34.8%, and the R value was 51.5% (r = Fobs−/ Fcalc/ Fobs), whereas the corresponding values for the highest background peak were 27.5 and 55.0%, respectively.

RESULTS

Cloning, Expression, and Purification of D. melanogaster dUTPase—The dUTPase gene is localized on chromosome 2L, as shown in Fig. 1A. Its closest neighbors include the genes for a galactose-binding lectin (53) and a putative nucleoprotein protein (54) upstream, as well as an accessory gland-specific protein (55) and a protein with unknown function downstream. Further inspection up to an 8-kb region proximal to the dUTPase on both DNA strands reveals 17 additional genes, encoding cytoskeletal proteins, metabolic enzymes, and stem cell renewal factors, together with 9 genes with not yet determined function gene products. No enzymes or other factors known to be involved in either DNA metabolism or repair can be localized to this region, except dUTPase. The ~1-kb region upstream of the dUTPase gene was investigated for presence of possible transcriptional regulatory sequences. The presence of a TATA box (TAATAA) and the Drosophila specific zeste factor site (CGAGGC) (56) was identified upstream of the start ATG codon (at locations −21 and −110, respectively).

The clone LD 20050 was introduced into pET vectors, and overexpression was conducted in different E. coli strains. Fig. 1B shows representative overexpression and purification results. Both constructs were used in the experiments at a purity of >95%. DNA sequencing performed on both strands gave perfect agreement with the original starting clone. The results of N-terminal sequencing and electrospray mass spectrometry of the purified proteins are given in Fig. 1C and Table I. The expressed proteins lack the N-terminal methionine residue, present in the genomic sequence, probably because of the methionine formylation-induced cleavage frequently observed in E. coli expression systems. When this loss is taken into account, measured molecular masses are in perfect agreement with the values calculated from the expected sequence (19,829 versus 19,826 and 17,199 versus 17,200 for the constructs 1–187 and 1–159, respectively). Electrospray mass spectrometry results of the peptide fragments of a total tryptic digest,
together with microsequencing and expected molecular mass data of the tryptic fragments given in Table I, identify the sequence of the expressed proteins with great certainty. As a most stringent criteria for protein purity, Fig. 1 demonstrates that single crystals suitable for x-ray diffractional analysis (cf. below) can be grown from the purified recombinant D. melanogaster dUTPase (1–159).

**D. melanogaster dUTPase Behaves as a Homotrimer in Solution**—Quaternary structure of D. melanogaster dUTPase constructs was determined using analytical gel filtration. The apparent molecular masses of the constructs 1–187 and 1–159 were calculated from the calibration curve as 65 and 54 kDa, respectively (data not shown). These values together with the apparent molecular mass estimated under denaturing conditions on SDS-PAGE gels (23 and 19 kDa) and measured by mass spectrometry (19,829 and 17,199 Da) identify the native proteins as homotrimeric oligomers. To confirm these results, dynamic light scattering experiments were also carried out on the construct 1–159 in 50 mM Tris, 50 mM NaCl, and 1 mM dithiothreitol pH 7.0 buffer at 4 and 20 °C (data not shown). Dynamic light scattering results estimated an apparent molecular mass of 51.5 kDa that further strengthens the homotrimeric organization of D. melanogaster dUTPase. These observations are especially important in view of the structural data on several EuBaR dUTPases (24–27) that show ultimate dependence of organization of the active site on the correct formation of the trimer. Having established that Drosophila dUTPase also forms homotrimers, kinetic investigations were initiated.

**Kinetic Characterization**—Ion exchange chromatography assays of the enzymatic activity showed that both constructs of D. melanogaster dUTPase are able to hydrolyze dUTP, dCTP, and dTTP to their corresponding monophosphate nucleotides with no detectable formation of diphosphate nucleotides as products of the reaction (data not shown). In addition to the fact that the /H9251-/H9252-phosphodiester bond is cleaved during the enzyme-catalyzed reaction of dUTP, no hydrolysis of dUDP was found to occur when incubating 10 mM dUDP with 0.1 mM dUTPase in 50 mM TES, 10 mM MgCl₂ pH 7.5 buffer for 48 h at room temperature. Kinetic parameters of the enzyme for dUTP and the close substrate analogues (dCTP, dTTP, and UTP) are...
reported in Table II, in comparison with one prokaryotic and one retroviral dUTPase.  

| Nucleotide | D. melanogaster dUTPase | EIAV dUTPasea | E. coli dUTPaseb |
|------------|------------------------|----------------|-----------------|
|            | $k_{\text{cat}}$ | $K_M$ | $k_{\text{cat}}/K_M$ | $k_{\text{cat}}$ | $K_M$ | $k_{\text{cat}}/K_M$ | $k_{\text{cat}}$ | $K_M$ | $k_{\text{cat}}/K_M$ |
| dUTP       | 12 (11) | 0.4 (0.45) | $3 \times 10^{-5}$ ($2.4 \times 10^{-5}$) | 25 | 1.1 | $2 \times 10^{7}$ | 7 | 0.2 | $3.5 \times 10^{7}$ |
| dTTP       | 0.1 | 640 | 156 | <0.5 | 260 | <2000 | ND | >20,000 | <100 |
| dCTP       | 0.7 | 2300 | 304 | <2 | <300 | <1000 | 0.6 | 4000 | <100 |
| UTP        | <0.05 | ND | ND | <0.3 | 2500 | <1000 |

a The values in parentheses have been determined for the full-length construct of D. melanogaster dUTPase.

b From Ref. 68.

c From Ref. 61.

d ND, not determined.

A further increase in metal ion concentration did not lead to a change in the kinetic parameters. Mg$^{2+}$-free conditions were checked by atomic absorption spectroscopy measurements. In addition, the concentration of Mn$^{2+}$, a divalent metal ion shown to closely mimic the co-factor effect of Mg$^{2+}$ on dUTPase (42), was also measured and was found to be undetectable. The significant catalytic efficiency of D. melanogaster dUTPase in the absence of Mg$^{2+}$ was unexpected and surprising. Enzymes involved in the metabolism of nucleotide phosphates and DNA or RNA usually require divalent metal ions as co-factors. Recently, it was shown that dUTPase from E. coli also possesses a similar catalytic activity in the absence of Mg$^{2+}$ (42). It seems that dUTPases in general might have a less stringent need for divalent metal ions in the continuous spectrophotometric activity assay. In the absence of Mg$^{2+}$, $k_{\text{cat}}$ decreased by 40%, and $K_M$ increased 4-fold as compared with the values measured in the presence of 5 mM Mg$^{2+}$. Components to ensure the lack of divalent metal ions in the continuous spectrophotometric activity assay. In the absence of Mg$^{2+}$, $k_{\text{cat}}$ decreased by 40%, and $K_M$ increased 4-fold as compared with the values measured in the presence of 5 mM Mg$^{2+}$.
Binding Affinity of D. melanogaster dUTPase toward dUDP and dUMP—The nonhydrolyzable character of dUDP by D. melanogaster dUTPase, as established in the previous section, identifies this nucleoside diphosphate as a close substrate analogue useful for detailed analysis of the active site in equilibrium complexation experiments. In previous studies from this laboratory, CD spectroscopy was found useful for exploring the interaction of dUTPase from E. coli with dUDP (30, 33, 39). Following these earlier observations, spectra were recorded for the D. melanogaster dUTPase enzyme, for dUDP, and for an equimolar mixture thereof (Fig. 2A). In the wavelength range 250–340 nm, a considerable enhancement of ellipticity, induced upon the complexation of the enzyme and dUDP, is evident when comparing the arithmetic sum of the spectra of the components measured alone with the spectrum measured in their mixture. The difference spectrum shows maximum at 270 nm. Titration experiments were carried out (Fig. 2B) to follow the difference ellipticity signal upon saturation of the enzyme with the ligand and to allow for an estimation of the dissociation constant and the number of binding sites. Similar experiments were carried out with the product dUMP. Dissociation constants calculated from the saturation curves of the enzyme with the ligand and to allow for an estimation of the dissociation constants calculated from the saturation curves of the enzyme-substrate interaction (K_M), have been recorded (Fig. 3).

The above titrations were conducted in the presence of 5 mM Mg^{2+}. Based on the results of the kinetic experiments where Mg^{2+} was shown to be a co-factor possibly strengthening the enzyme-substrate interaction (K_M decrease), titrations were also carried out in the absence of Mg^{2+} (Fig. 2B). Lack of the divalent metal ion caused a 4-fold increase in the dissociation constant of the dUDP-dUTPase complex.

Using a kinetic approach, inhibitory constants (K_I) of dUDP and dUMP were also determined. The enzyme reactions were followed by the continuous spectrophotometric assay in the presence of varying concentrations of dUDP and dUMP. The results were plotted following Webb’s equation (58), assuming that dUMP and dUDP are competitive inhibitors with respect to dUTP. The data are presented in Table III.

In the above measurements, the two constructs of D. melanogaster dUTPase behaved the same way and produced the same quantitative results. Table III compiles both K_M and K_I values for D. melanogaster dUTPase, determined in the present study, as well as for E. coli enzyme. The fact that the dissociation constants and the inhibition constants are in close agreement indicates the existence of rapid equilibria between enzyme and substrate as well as enzyme, and the substrate analogous inhibitors. Mg^{2+} greatly enhances complex formation between the nucleotide ligands and either E. coli or D. melanogaster dUTPases. The binding affinity of the D. melanogaster enzyme toward dUDP and especially toward dUMP is considerably higher as compared with that of the E. coli enzyme. The increased affinity of dUMP to the D. melanogaster enzyme indicates that the binding interactions with the product of the enzymatic reaction are more pronounced. To characterize these interactions with respect to enzyme conformation, further spectroscopic and thermodynamic experiments were designed.

Ligand-induced Conformational Changes—Similarities in kinetic behavior toward the physiological substrate among bacterial, retroviral, and D. melanogaster dUTPases, in addition to the sequence conservation (Fig. 1 and Table II), suggested similar structural characteristics. To check this suggestion, investigations were carried out to characterize protein structure at different levels. CD spectra in the 190–240-nm wavelength range, known to reflect secondary structural content with reasonable accuracy (59), have been recorded (Fig. 3). D. melanogaster dUTPase constructs 1–187 and 1–159 show very
similar CD spectra in the far-UV wavelength range, characterized with a monotonous decrease to 200 nm followed by an increase up to 190 nm. Quantitative evaluation of the spectra by the k2d program (60) resulted in estimates for secondary structural content (α, 8 ± 1%; β, 45 ± 3%; other, 47 ± 2%), revealing an overwhelming majority of β-structure over α-helices in the folded protein. These values are very close to those estimated from the spectrum of the E. coli enzyme (α, 11%; β, 42%; other, 47%) (33), which were also confirmed by the crystal structure (26).

Following the previous observations with regard to the cofactor role of Mg\(^{2+}\) in catalysis and dUDP/dUMP binding, we also tested whether Mg\(^{2+}\) has any structural role that may be reflected in CD measurements. Fig. 3 shows that a significant enhancement of the negative CD spectral signals in the 200–240-nm wavelength range is induced by the addition of the metal ion at a saturating concentration of 5 mM. There is no such metal ion-induced change in the case of the E. coli dUTPase (30). It is important to emphasize that this effect of Mg\(^{2+}\) did not require the presence of the nucleotide.

To decide whether nucleotide ligands induce further conformational changes, dUDP was added to the Mg\(^{2+}\)-containing dUTPase solution, and far-UV CD spectra were recorded. The concentration of the nucleotide was set at levels to assure practically 100% complexation of the enzyme based on the dissociation constants determined in the previous section. As shown on Fig. 3, dUDP induced significant further ellipticity enhancement, reflecting some conformational change. Enhancement of the negative ellipticity signal in the wavelength range characteristic for peptide bond conformations argues for an increment in the ordered conformation upon binding of dUDP. It is worthwhile to point out that in similar experiments with E. coli dUTPase, no such conformational change could be detected upon complexation of dUDP with the enzyme (30).

In addition to the results obtained by CD spectroscopy, a further experimental technique was sought to study ligand-induced conformational effects. DSC provides direct quantitative thermodynamic data on protein stability, a sensitive measure of conformation and also indicates the oligomeric status of the protein during thermal unfolding. Microcalorimetric scans of both D. melanogaster dUTPase constructs and E. coli dUTPase are shown in Fig. 4. Similar measurements were conducted with the E. coli dUTPase. All of the scans could be fitted to model curves, assuming reversible two-state transitions, with a single cooperative step of heat absorption. Thermodynamic parameters calculated from best fitted model curves are given in Table IV. It is evident from the thermodynamic parameters calculated from the thermograms that melting of the long construct D. melanogaster dUTPase 1–187 is not associated with higher melting temperature or a larger melting enthalpy as compared with the short construct 1–159. Both D. melanogaster dUTPase samples have transition temperatures of 53 °C, some 20 °C lower than the transition temperature of the E. coli dUTPase. Table IV shows the calculated van’t Hoff and calorimetric enthalpies and their ratios, as a sensitive test for reversibility of the melting and also for the nature of the oligomeric entity undergoing the transition (45).

van’t Hoff enthalpies depend only on the mid-point of transition and the height of the heat absorption curve and assume 100% reversibility and two-state transition with no allowance

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**TABLE III**

Dissociation and inhibition constants of the substrate analogues dUDP and dUMP toward D. melanogaster and E. coli dUTPase.
The standard errors were 18% and 32% for determination of $K_d$ and $K_I$ values, respectively.

| Enzyme                        | dUDP $K_d$ $\mu M$ | dUDP $K_I$ $\mu M$ | dUDP $K_d$ $\mu M$ | dUDP $K_I$ $\mu M$ |
|-------------------------------|--------------------|--------------------|--------------------|--------------------|
| D. melanogaster dUTPase (1–187) | 210                | 48.0               | ND                 | 55                 |
| D. melanogaster dUTPase (1–159) | 185.4              | 40.2               | ND                 | 65.4               |
| E. coli dUTPase               | ND                 | ND                 | ND                 | 1500$^c$           |

$^a$ Values determined via differential CD spectroscopy in the present study.

$^c$ ND, not determined.

From Ref. 2.

$^d$ From Ref. 39.

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**Fig. 3.** Far-UV CD analysis of Mg\(^{2+}\)- and dUDP-induced structural effects. Far-UV circular dichroism spectra of D. melanogaster dUTPase in the absence of Mg\(^{2+}\) (thin solid line), in the presence of 5 mM Mg\(^{2+}\) (thick solid line), in the presence of 5 mM Mg\(^{2+}\) and 100 µM dUDP (dashed line) and in the presence of 5 mM Mg\(^{2+}\) and 1 mM dUMP (dotted line), are shown.
for intermediates. Calorimetric enthalpies equal the area under the heat absorption curve; molar calorimetric enthalpies are calculated by taking into account the molar concentration of the sample. The ratio of van’t Hoff and calorimetric enthalpies gave a close approximation of $\frac{3}{2}$ when using the monomer concentration. When calculating the concentration of the protein sample in trimers, the ratios are very close to unity. These results indicate that dUTPase, either from E. coli or from D. melanogaster, unfolds upon heating as a trimeric folding unit, with no thermodynamically significant population of folded monomers.

The presumed structural role of Mg$^{2+}$ and nucleotide ligands (cf. Fig. 3) was also investigated in the microcalorimetric experiments. The presence of Mg$^{2+}$ induces a slight but significant increase in the melting temperature of D. melanogaster dUTPase (Fig. 4 and Table IV). In separate heating cycles, dUMP or dUDP was added to Mg$^{2+}$-containing D. melanogaster dUTPase, and resulting complex was subjected to thermal unfolding. The results indicate that both nucleotides induce further increments in the melting temperature.

CD and DSC experiments consonantly indicate that binding of Mg$^{2+}$ to D. melanogaster dUTPase modulates the protein conformation even in the absence of nucleotide ligands. dUMP and dUDP binding to the enzyme in the presence of Mg$^{2+}$ induce further enzyme conformational changes that can be followed by both techniques. To localize the nucleotide-induced conformational effects within the protein, limited proteolysis experiments were designed.

Limited trypsinolysis maps the structural hierarchy of the trimeric protein and provides information on possible flexible segments. The first set of experiments was therefore carried out with both constructs 1–187 and 1–159 in the absence of nucleotides. The time course of proteolysis as followed by SDS-PAGE and enzyme activity measurements is shown in Fig. 5 (A and B, respectively). In the case of the full-length construct 1–187, a slight decomposition, cause by a storage of 6 months at $-70\,^\circ\text{C}$ and two cycles of freeze/thaw, is seen even before the addition of trypsin (Fig. 5A, lane 11). The specific activity of the enzyme was not perturbed by this degradation. The site of this cleavage, considered to be the most sensitive proteolytic site, was identified by mass spectrometry and blot microsequencing as a composite of the tryptic sites Lys$^{12}$, Lys$^{13}$, and Lys$^{15}$, leading to the loss of the N-terminal 12–15 residues. The next most sensitive site in the case of the 1–187 construct was shown to be present at Lys$^{52}$, leading to the loss of the C-terminal fly-specific Ala-Pro-rich extension. The upper band in Fig. 5A (lane 12) corresponds to the 16–159 species, as shown by mass spectrometry (molecular mass is 15,599 Da, in perfect agreement with the sequence), and blot microsequencing (N-terminal sequence is Ile-Asp-Thr-Xaa-Val). Cleavage at this site is completed in less than 5 min, both in the absence (data not shown) and in the presence of dUDP (Fig. 5A, lane 12). Limited trypsinolysis of the 1–159 construct consists of two steps easy to distinguish under the experimental conditions. The first rapid cleavage is completed in 5 min (Fig. 5A, lanes 1 and 2), and leads to the loss of the N-terminal 15 residues (as confirmed by blot microsequencing). The second slower step leads to a further mass decrease of similar proportions. The final resulting fragment from both constructs runs to the very same position on SDS-PAGE (Fig. 5A, lanes 4 and 12, lower bands), corresponding to a molecular mass of $-14\,\text{kDa}$. Electrospray mass spectrometry of this purified fragment from both constructs gave the same molecular mass of 14,609 Da. Blot microsequencing data indicated that the N terminus of the 14,609-Da fragment is Ile-Asp-Thr-Cys-Val, which unanomously identifies this 14,609-Da tryptic fragment as the peptide of residues 16–148, which has a calculated mass of 14,606.6 Da (Fig. 1B for the amino acid sequence). This fragment was analyzed by gel filtration chromatography to reveal an approximate native molecular mass of 43 kDa. Comparison of the subunit molecular mass of 14 kDa from SDS-PAGE and mass spectrometry and the native molecular mass from analytical gel filtration argues for the trimeric quaternary structure of the trypsinolysed fragment. Enzymatic activity of the purified 16–148 fragment is drastically reduced; $k_{\text{cat}}$ is 1.5 ± 0.5% of that of the native enzyme.

The above results identified three or two sensitive trypsic sites in the longer 1–187 or the shorter 1–159 constructs of D. melanogaster dUTPase, respectively (Fig. 5C). To probe conformational changes at these sites induced by binding of nucleotide ligands, trypsinolysis experiments have been carried out in the presence of the nonhydrolyzable substrate analogue dUDP and the product dUMP. The results show that trypsinolysis of the longer 1–187 construct quickly leads to the loss of the specific C-terminal fragment, which distinguishes the 1–187 construct from the 1–159 construct, both in the absence and in the presence of dUDP (Fig. 5A, lanes 11 and 12). Further investigations with the nucleotide ligands were therefore restricted to the 1–159 construct. Lanes 5–7 and 8–10 in Fig. 5A, in comparison with lanes 2–4, show that on one hand both dUDP and dUMP have no significant effect on the first tryptic cleavage at Lys-15 of the N terminus, whereas on the other hand they have a robust protective effect on the tryptic cleavage at Arg$^{146}$ of the C terminus. The process of limited trypsinolysis and the protective effects of the nucleotide ligands are schematically summarized in Fig. 5C.

The time course of the tryptic digestion was followed by enzyme activity measurements as well. The graphs presented in Fig. 5B show the loss of enzymatic activity caused by an apparent first order reaction with rate constants of 0.0096, 0.0048, and 0.0046 min$^{-1}$ for D. melanogaster dUTPase (1–159) in the absence of nucleotide ligands, in the presence of dUMP, and in the presence of dUDP, respectively. In contrast to the results obtained by SDS-PAGE analysis of the progress of proteolysis, there is no sign of the activity decrease being biphasic under the same experimental conditions. In the first 5 min, corresponding to practically complete cleavage at the N terminus, there is no significant activity decrease. Rather, the rate constants of the activity decrease time courses are in
qualitative agreement with the time course of the tryptic cleavage at Arg148, as it may be judged from the gel electrophoretogram in Fig. 5A.

The limited proteolysis experiments indicate that the peptide bonds connecting both the N- and the C-terminal flanking regions of the enzyme (15 and 28 residues, respectively) are highly exposed, suggesting that these regions do not contain ordered structural elements and are not deeply involved in the protein fold. Consonantly, the presence of the C-terminal 28-residue segment in the enzyme does not cause appreciable CD spectral enhancement, neither an increment in the melting temperature measured by DSC, which underlines the conclusion of the flexible, mostly unstructured character of this segment. The N- and C-terminal flanking regions do not contain any of the five conserved motifs characteristic for all dUTPases and can be cleaved from the enzyme without any loss of catalytic activity. A further, rather sensitive tryptic cleavage site is situated at the beginning of conserved motif 5, right after the strictly conserved Arg 148 residue. Cleavage at this peptide bond leads to a loss of enzyme catalytic activity but retention of the homotrimeric structure. Significant protection against tryptic cleavage at motif 5 segment is provided by both the enzymatic

qualitative agreement with the time course of the tryptic cleavage at Arg 148, as it may be judged from the gel electrophoretogram in Fig. 5A.

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product dUMP and the nonhydrolyzable analogue dUDP. The protective effect of these two nucleotides indicates that the conformational changes induced by their binding to the *D. melanogaster* dUTPase (Figs. 3 and 4) necessarily involve the motif 5-containing segment of the protein. The situation with respect to tryptic cleavage at the motif 5 conserved arginine leading to inactivation is very similar to the case of the *E. coli* enzyme. However, in sharp contrast to the *D. melanogaster* dUTPase trypsinolysis, no protection against trypsinolysis of the bacterial enzyme could be observed in the presence of either dUMP or dUDP (30, 33).

**Crystallization and Preliminary X-ray Analysis Results**—Because several independent approaches in the solution phase detailed in the previous sections indicated that *D. melanogaster* dUTPase conformational flexibility might be different as compared with the *E. coli* enzyme, preliminary crystallographic experiments were initiated to decide whether such conformational differences might also exist in the crystal phase. The crystals were grown from the short construct containing the first 159 residues of the *Drosophila* dUTPase (Figs. 3 and 4) necessarily involve the motif 5-containing segment of the protein. The situation with respect to tryptic cleavage at the motif 5 conserved arginine leading to inactivation is very similar to the case of the *E. coli* enzyme. However, in sharp contrast to the *D. melanogaster* dUTPase trypsinolysis, no protection against trypsinolysis of the bacterial enzyme could be observed in the presence of either dUMP or dUDP (30, 33).

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**DISCUSSION**

In the recently completed annotation of the *D. melanogaster* genome, there is a single dUTPase gene on chromosome 2L in the 32D1 segment. The fact that the *D. melanogaster* dUTPase gene has no introns precludes the mechanism of alternative exon usage, shown to direct the production of isofom-specific transcripts of the human gene, from generating different isoforms of the enzyme. Human dUTPase, the only case where isoforms have been described, has one nuclear and one mitochondrial isoform, with the latter possessing a mitochondrial transport signal and processing site (22). No such mitochondrial transport signal sequence is found at the N terminus of *D. melanogaster* dUTPase. Experiments to identify the physiological isoforms of fly dUTPase are in progress in our laboratory. Both the N and C termini of fly dUTPase may contain subcellular localization segments sensitive to proteolytic cleavage (Fig. 5), which varies from the exposed character and accessibility that might be helpful in binding to potential transport proteins.

One of the aims of the present study was to decide whether the *Drosophila*-specific C-terminal 28-residue extension might have some regulatory effect on enzyme kinetic behavior. The
results obtained with the purified recombinant protein constructs reveal that no such effect could be observed in vitro. It is, of course, possible that this region has some potential other role, not accessible in the present experiments conducted in purified systems. Such a role might be to offer a recognition site for some cellular interacting partners, similar to the case of the rat enzyme with an N-terminal species-specific extension that binds to peroxisome proliferator-activated receptor α (62).

Conformational Shifts in the C Terminus Are Induced Differently in Drosophila and E. coli dUTPases—Detailed studies from several laboratories unanimously indicated that the ordering of the C terminus of E. coli dUTPase requires interaction with the complete triphosphate chain of the substrate (28, 30, 34). In the present work, experimental evidence from limited proteolysis, CD spectroscopy, and DSC argue in agreement that dUMP and dUDP are also capable of inducing a significant conformational change (an increment in ordered protein conformation) upon binding to D. melanogaster dUTPase. Limited proteolysis experiments localized this conformational change to the C-terminal conserved motif 5, removal of which leads to inactivation of the enzyme. Three-dimensional crystal structures of the human and the feline immunodeficiency virus dUTPase in complex with dUTP analogues suggest that the ordered conformation of the C terminus is realized by its closing over the active site and contacting the bound nucleotide phosphate (24, 29, 63). The present results therefore show for the first time that formation of the catalytically competent closed enzyme conformer in solution (i.e. in the absence of any artifacts possibly resulting in ordering because of specific crystallization conditions) may occur with altered timing in the catalytic cycle of dUTPases from different evolutionary origin.

In contrast to E. coli dUTPase, the D. melanogaster enzyme is shown to adopt the closed conformer with the product dUMP. Structural characteristics of this conformational shift are analyzed in detail in the following study by multidimensional NMR spectroscopy.

The capability of mono- and diphosphates of deoxyuridine to induce the conformational shift of the C-terminal region of fly dUTPase, in contrast to the E. coli enzyme, indicates some alteration in the pattern of binding interactions. This alteration is shown to be reflected in the drastic increase of binding affinity of fly dUTPase toward dUMP. The affinity toward dUDP is also significantly increased, whereas the Michaelis constant toward dUTP is comparable with that observed with the E. coli enzyme (Tables II and III).

The Divalent Metal Ion as a Structural Co-factor in Eukaryotic dUTPases—CD and DSC experiments indicated that Mg$^{2+}$ binding to Drosophila dUTPase induces protein conformational changes even in the absence of nucleotide ligands. These results argue for a metal ion site present in the fly enzyme that is accessible in the nucleotide-free protein. We suggest that apart from its universal role of a catalytically important co-factor for dUTPases from all sources, Mg$^{2+}$ ion may also have an additional structural role in some of the dUTPases. Two dUTPase crystal structures have been reported where divalent metal ions (Mg$^{2+}$ in both cases) were identified at locations far away from the active site. In the crystal structure of dUTPase from feline immunodeficiency virus (FIV), the metal ion is found within the central channel of the trimer (Fig. 6A) (25). In this niche, the metal ion coordinates to aspartate residues and to structural water molecules that provide six ligands in the metal coordination sphere in the hexagonal geometry that is generally preferred for divalent metal ions in proteins (64). In the structure of dUTPase from equine infectious anemia virus, a close lentiviral relative of FIV lentivirus, however, no such Mg$^{2+}$ binding is observed. The central channel of this retroviral enzyme is strongly apolar, and there are no such residues present, which may provide coordination environment for divalent metal ions. Moreover, amino acid sequence conservation patterns in all retrovir, except for the feline immunodeficiency virus dUTPase case, as well as in other dUTPase sequences fail to locate any residues capable of metal coordination at the site observed exclusively for the FIV dUTPase (31). These findings argue that the FIV dUTPase Mg$^{2+}$-binding site within the central channel is a species-specific site.

In the human enzyme, Mg$^{2+}$ is localized also within the central channel but at a distance of 16 Å from the site in FIV dUTPase (Fig. 6A). The metal ion coordinates to three glutamate side chain (one from each subunit) oxygens and one water. The glutamate residue providing coordination to Mg$^{2+}$ is strictly conserved in all eukaryotic dUTPases (31). The coordination geometry agrees well with a theoretical tetrahedron (Fig. 6B) that strengthens the conclusion that only four coordinating ligands are present in this case. This coordination is rather unusual for Mg$^{2+}$ sites in proteins but is also found for the site present in the ATPase subunit of a maltose transporter (65). Our present results of both CD spectroscopy and microcalorimetric experiments consonantly indicated significant effects of Mg$^{2+}$ binding to Drosophila dUTPase. We propose that the observed conformational effects of Mg$^{2+}$ on Drosophila dUTPase is due to metal binding to this intersubunit channel site that is 18 Å away from the nucleotide accommodating active site.

Fly dUTPase Can Be Crystallized in the Monomeric Form—To account for the presence of enzyme monomers in the crystal phase, two possibilities may be considered. First, the 2-propanol solvent molecules, present at high concentration in the crystallization medium, may serve as ligands of adequate polarity for the amino acid residues on that specific surface of the subunit that otherwise generally participates in subunit interactions. In agreement with this possibility, alcohols are reported to modulate protein folding (66, 67). The fact that in the human enzyme crystal structure the protein still forms a trimer may be due to the differences in the crystallization condition (the human enzyme was precipitated with sodium citrate at pH 7.5–8.5). Second, the acidic pH of the crystallization solvents might perturb Mg$^{2+}$ binding to the enzyme by titrating the carboxylate groups into their acidic form. Lack of coordination capacity for Mg$^{2+}$ within the inner 3-fold channel
of the trimer may contribute to an increased instability of the trimer, because evidence from CD and DSC experiments con-
siderably indicates a structural role for the metal ion. An in-
esting question regarding the presence of the monomer in the
solution phase is put forward by these crystallization results.
Dynamic light scattering experiments in the solution phase
simulating the environment in the crystallization medium in-
dicated that the solution phase contained only a hardly detect-
able amount of the protein, but that was found to be in the
monomeric form. This is not very surprising because the suc-
cess of crystallization means that the crystallization conditions
efficiently drive precipitation. The lack of trimeric organization
may therefore be expected to occur only under specific circum-
stances and in dUTPsases that have an increased polarity of
subunit interactions, i.e. those of the eukaryotic subfamily (31).
It was so far impossible to obtain the monomer of a homotrimer-
ic dUTPase. The present crystallization renders this rare and
interesting molecular species for further high resolution struc-
tural investigations. The three-dimensional structure of this
dUTPase monomer is expected to greatly advance our knowl-
edge about the basic thermodynamic forces that drive forma-
tion of the intertwined trimer with its intricate subunit
interactions.

Conclusions—Major differences were identified between bac-
terial and eukaryotic representatives of homotrimeric dUTP-
ases. Among these, the possibility of retaining the flexible
C-terminal motif 5 in an ordered conformation in the presence of
the product of the enzymatic reaction is especially intriguing,
because it basically changes the schedule of conformational
events in the catalytic cycle. It was therefore of interest to
attack this problem via high resolution structural studies in
solution. The resulting multidimensional NMR spectroscopic
investigations are reported in the following study.

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REFERENCES
1. Shlomai, J., and Kornberg, A. (1978) J. Biol. Chem. 253, 3305–3312
2. Larsson, G., Nyman, P. O., and Kvassman, J. O. (1996) J. Biol. Chem. 271,
24010–24016
3. Kornberg, A., and Baker, T. A. (1991) DNA Replication, 2nd Ed., Freeman,
New York
4. Tye, B. K., Nyman, P. O., Lehman, I. R., Hochhauser, S., and Weiss, B. (1977)
Proc. Natl. Acad. Sci. U. S. A. 74, 154–157
5. Ingraham, H. A., Dickey, L., and Goulain, M. (1986) Biochemistry 25,
3225–3230
6. Pearl, L. H., and Savva, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1069–1075
7. el-Hajj, H. H., Zhang, H., and Weiss, B. (1988) J. Biol. Chem. 263, 1069–1075
8. Gadsden, M. H., McIntosh, E. M., Game, J. C., Wilson, P. J., and Haynes, R. H.
J. Virol. 69, 2767–2776
9. Webb, J. L. (1963) J. Biol. Chem. 238, 108–111
10. Blumenthal, R., and Kornberg, A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75,
6016–6020
11. Traut, T. W. (1994) Biochemistry 33, 6556–6563
12. Vallone, P., and Kornberg, A. (1980) Biochemistry 19, 3046–3054
13. Siddiqui, S. H., and Kornberg, A. (1978) J. Biol. Chem. 253, 4108–4115
14. Kornberg, A., and Lehman, I. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78,
738–742
15. Mosti, M., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80,
1529–1533
16. Baraba, N., and Kornberg, A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75,
6021–6025
17. Weiss, B., and Kornberg, A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77,
4825–4829
18. Goodfellow, P., and Kornberg, A. (1978) Cell 14, 635–644
19. Giroir, L. E., and Deutsch, W. A. (1987) J. Biol. Chem. 262, 130–134
20. Dauter, Z., Persson, G., Vandeputte, C., Bergman, A. C., Persson, R., and
Nyman, P. O. (1998) FEBS Lett. 421, 83–88
21. Fiser, A., and Sverny, S. (1998) Biochim. Biophys. Acta 297, 543–549
22. Martin, R. M. (1996) J. Biol. Chem. 271, 7752–7757
23. Dauter, Z., and Persson, S. (1997) J. Biol. Chem. 272, 6972–6980
24. Tinklenberg, B. A., Fazzone, W., Lynch, F. J., and Dauter, R. D. (2003) Exp.
Cell Res. 297, 39–46
25. Mol, C. D., Harris, J. M., McIntosh, E. M., and Tainer, J. A. (1996) Structure
4, 1077–1092
26. Lerner, D. L., Wagaman, P. C., Phillips, T. R., Prospero-Garcia, O., Henricken,
S. J., Fox, H. S., Bloom, F. E., and Elder, J. H. (1995) Proc. Natl. Acad.
Sci. U. S. A. 92, 7480–7484
27. Dauter, Z., and Persson, S. (1997) J. Biol. Chem. 272, 7752–7757
28. Tinklenberg, B. A., Fazzone, W., Lynch, F. J., and Dauter, R. D. (2003) Exp.
Cell Res. 297, 39–46
29. Giroir, L. E., and Deutsch, W. A. (1987) J. Biol. Chem. 262, 130–134
30. Dauter, Z., and Persson, S. (1997) J. Biol. Chem. 272, 6972–6980
31. Tinklenberg, B. A., Fazzone, W., Lynch, F. J., and Dauter, R. D. (2003) Exp.
Cell Res. 297, 39–46
32. Martin, R. M. (1996) J. Biol. Chem. 271, 7752–7757
33. Dauter, Z., and Persson, S. (1997) J. Biol. Chem. 272, 6972–6980
34. Tinklenberg, B. A., Fazzone, W., Lynch, F. J., and Dauter, R. D. (2003) Exp.
Cell Res. 297, 39–46
35. Fiser, A., and Sverny, S. (1998) Biochim. Biophys. Acta 297, 543–549
36. Martin, R. M. (1996) J. Biol. Chem. 271, 7752–7757
37. Dauter, Z., and Persson, S. (1997) J. Biol. Chem. 272, 6972–6980
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