The complete triphosphate moiety of non-hydrolyzable substrate analogues is required for a conformational shift of the flexible C-terminus in *E. coli* dUTP pyrophosphatase

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Abstract The molecular mechanism of substrate analogue interaction with *Escherichia coli* dUTPase was investigated, using the non-hydrolyzable 2′-deoxyuridine 5′-(α,β-imido)triphosphate (α,β-imido-dUTP). Binding of this analogue induces a difference in the far UV circular dichroism (CD) spectrum arguing for a significant change in protein conformation. The spectral shift is strictly Mg^{2+}-dependent, does not appear with dUDP instead of α,β-imido-dUTP and is not elicited if the flexible C-terminal arm is deleted from the protein by limited tryptic digestion. Involvement of the C-terminal arm in α,β-imido-dUTP binding is consistent with the finding that this analogue protects against tryptic hydrolysis at Arg-141. Near UV CD of ligand-enzyme complexes reveals a characteristic difference in the microenvironments of enzyme-bound dUDP and α,β-imido-dUTP, a difference not observable in C-terminally truncated dUTPase. The results suggest that (i) closing of the active site during the catalytic cycle, through the movement of the C-terminal arm, requires the presence of the complete triphosphate moiety of the substrate in complex with Mg^{2+}, and (ii) after catalytic cleavage the active site pops open to facilitate product release.

Key words: dUTP pyrophosphatase; Non-hydrolyzable substrate analogue; Circular dichroism spectroscopy; Flexible C-terminal arm; Motif 5; *Escherichia coli*

1. Introduction

DNA repair is indispensable for faithful conservation of genetic information. The ubiquitous enzyme dUTP pyrophosphatase (dUTPase, EC 3.6.1.23), catalyzing the hydrolysis of dUTP to dUMP and PP_i [1], plays a preventive role in this task by effectively reducing the dUTP/dTTP ratio to suppress ruinous incorporation of uracil into DNA [2]. Highly uracil-substituted DNA is subjected to excessive excision repair leading to double strand breaks and, eventually, cell death. dUTPase null mutations are either lethal (in *Escherichia coli* [3] and yeast [4]) or restrict the host range of several viruses [5,6], thereby proposing the enzyme as a target for drug design against invading organisms (bacteria, fungi or viruses). Drug design against the bacterial or viral proteins should rely on species-specific structural and/or mechanistic differences of dUTPases from host and invader.

Most dUTPases are homotrimers with three active sites located at the interfaces between the subunits [7]. Each active site is constructed by conserved sequence motifs [8] from different subunits. The C-terminal arm, containing the conserved Motif 5, is disordered in the crystal structures of the unliganded *E. coli* and human enzyme [7,9]. However, an interesting species-specific difference can be identified when comparing the liganded enzyme complex structures [7,10]. Upon dUMP, dUDP or dUTP binding to the human enzyme a large part of the C-terminus becomes ordered and visible in the electron density map while it is still disordered in the *E. coli* dUTPase-dUDP complex structure [7,10]. It is to be noted that all these complexes are devoid of metal ions, although Mg^{2+} is an important cofactor of the enzyme [11,12].

Recently, in an attempt to identify a functional role for the C-terminus in the bacterial enzyme, we deleted its last 11 residues comprising the main part of Motif 5 [13]. The cleavage leads to a dramatic decrease in catalytic activity but no detectable change in the affinity towards MgdUDP [13]. These data suggest that the factor responsible for the activity loss may, at least partly, reside in an impaired interaction between the γ-phosphate of the substrate and the truncated enzyme. A non-hydrolyzable dUTP analogue (2′-deoxyuridine 5′-(α,β-imido)triphosphate (α,β-imido-dUTP)), containing the complete α-β-γ phosphate chain, was synthesized recently [14], offering a convenient way for analysis of ligand-induced conformational changes in dUTPase. In the imido analogue the bridging oxygen between the α and β phosphorus atoms is replaced by an imido group, rendering the scissile bond non-hydrolyzable.

In this study the interactions of α,β-imido-dUTP and dUDP with native and C-terminally truncated dUTPase were investigated using far and near UV circular dichroism (CD) spectroscopy. These compounds are known to be strong competitive inhibitors [15]. Experiments were designed to provide an explanation for the fact that dUDP is not hydrolyzed by the enzyme, although it contains the scissile α-β phosphoanhydride bond. The results obtained are interpreted in the light of the available crystal structures and a molecular model is constructed for the catalytic cycle.

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Abbreviations: α,β-imido-dUTP, 2′-deoxyuridine 5′-(α,β-imido)triphosphate; dUTPase, dUTP pyrophosphatase (EC 3.6.1.23); CD, circular dichroism

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2. Materials and methods

2.1. Materials

dUTP, Q-Sepharose and Sephacryl S-200 were purchased from Pharmacia, Sweden. dUDP, Tes and HEPES buffers were obtained from Sigma, US. Phenol red indicator was from Merck, Germany. α,β-Imido-dUTP was synthesized as described previously [14]. All other materials were of analytical grade purity.

2.2. Enzyme preparation and assay

dUTPase from *E. coli* was purified as described previously [11]. The purified preparation appeared as a single band on SDS-PAGE when investigated by laser densitometry (see below), suggesting at least 98% purity. Protein concentration was measured spectrophotometrically using $A^{0.1\%}_{1cm,280nm} = 0.52$ [12]. Molecular mass of the trimeric *E. coli* enzyme is 49 kDa [9]. Throughout the present study, molar enzyme concentrations refer to the monomeric species. Before use, the enzyme was dialyzed against respective buffers. Enzyme activity was routinely assayed at enzyme concentrations of 25–50 nM in 40 μM dUTP, 1 mM $MgCl_2$ (providing saturating excess of Mg^{2+}), 150 mM KCl, 40 μM Phenol red, 1 mM TES/HCl pH 7.5 buffer (assay buffer). Proton release during the hydrolysis of dUTP was followed at 559 nm at 25°C [12], using Hewlett-Packard 8451A or JASCO V550 spectrophotometers and 10-mm path-length thermostatted cuvettes. Initial velocity was determined from the slope of the initial part (first 10 s) of the progress curve. The catalytic constant k_{cat} of native dUTPase was determined to be 5 s^{-1}, which is in accordance with previous investigations [13,15].

2.3. Tryptic digestion

Limited tryptic digestion of *E. coli* dUTPase and purification of the larger fragment were performed as previously described [13]. The tryptic larger fragment was at least 95% pure as judged from SDS-PAGE on 16% gels [16]. Protein bands were visualized by Coomassie brilliant blue R-250 staining and quantitative analysis was done by densitometry on a GelDoc densitometer (Bio-Rad).

2.4. CD measurements

Near UV (230–350 nm) or far UV (200–260 nm) CD spectra were recorded on a JASCO 720 spectropolarimeter using 10-mm or 1-mm path-length cuvettes, respectively, thermostatted to 25°C. Native or truncated dUTPase was titrated by stepwise addition of α,β-imido-dUTP or dUDP in 20 mM potassium-phosphate–1 mM $MgCl_2$, pH 7.5 buffer. Spectra measured immediately after mixing enzyme and ligand were unchanged for at least 30 min, suggesting that complexation requires less than 5 min under our experimental conditions. Three scans of each spectrum were averaged. Difference spectra were calculated by subtracting the spectra of the protein and the ligand measured alone from the spectrum measured in their mixture by using the built-in software of the spectropolarimeter.

3. Results

3.1. Binding of α,β-imido-dUTP induces a major protein conformational change of E. coli dUTPase

Far UV CD spectra of enzyme-ligand complexes are presented in Fig. 1. Addition of α,β-imido-dUTP to the enzyme significantly enhances negative ellipticity, arguing for a protein conformational change upon binding of the ligand (difference spectra Fig. 1B, solid line). This change is specific for the triphosphate analogue and is not observed with dUDP (Fig. 1B, dashed line). The altered modes of binding of tri- and diphosphate ligands are also reflected in the near UV CD difference spectra (Fig. 2B). While binding of α,β-imido-dUTP leads to a negative difference peak at 260 nm (solid line), that of dUDP elicits a positive peak at 270 nm (dashed line, cf. also [12,13]). Fig. 1AFig. 2A show the measured spectra of the protein or the ligand alone and the spectra of their mixture. The ligands show similar near UV CD spectra, with a positive peak at 270 nm (Fig. 2A and [12]). Since the difference peak elicited with dUDP is found at the same wavelength, it probably reflects the change in the optical activity of the ligand upon binding. However, the major difference peak elicited with α,β-imido-dUTP is situated at 260 nm (Fig. 2B, solid line) where neither the ligand nor the protein show appreciable CD signal of their own. This wavelength is the characteristic absorbance maximum for both phenylalanine and uridine, thus the 260 nm difference CD peak may

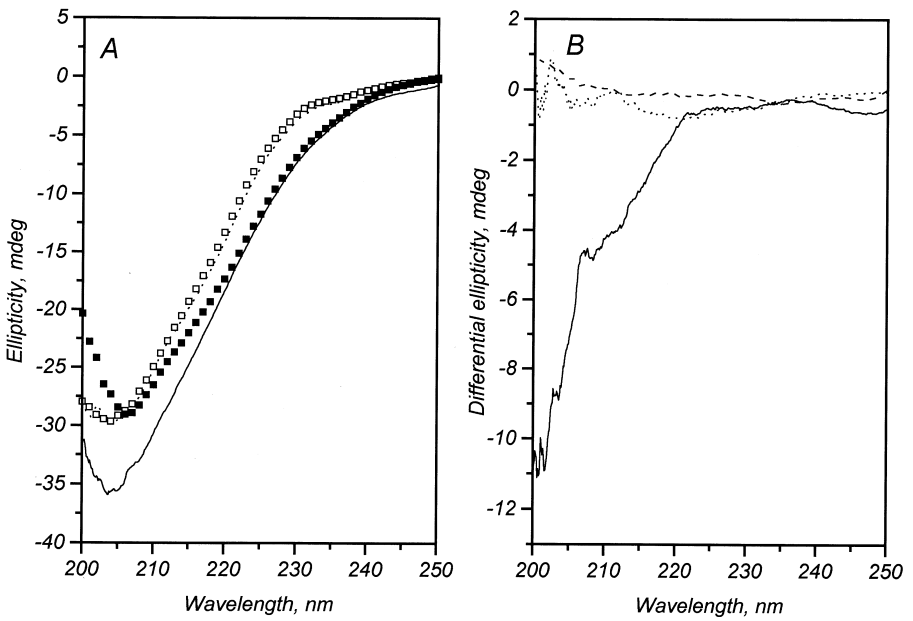


Fig. 1. Effects of α,β-imido-dUTP and dUDP on far UV CD spectrum of *E. coli* dUTPase. A: Spectra of native (closed symbols) or truncated (open symbols) enzyme alone (scattered graphs), and in mixture with α,β-imido-dUTP (line graphs, solid and dotted lines, respectively). B: Difference spectra of native dUTPase with α,β-imido-dUTP (solid line) or dUDP (dashed line). The dotted line is the difference spectrum of truncated dUTPase with α,β-imido-dUTP. Protein and ligand concentrations were 12.5 μM and 50 μM, respectively. Neither ligand displayed far UV CD signal of its own (not shown).

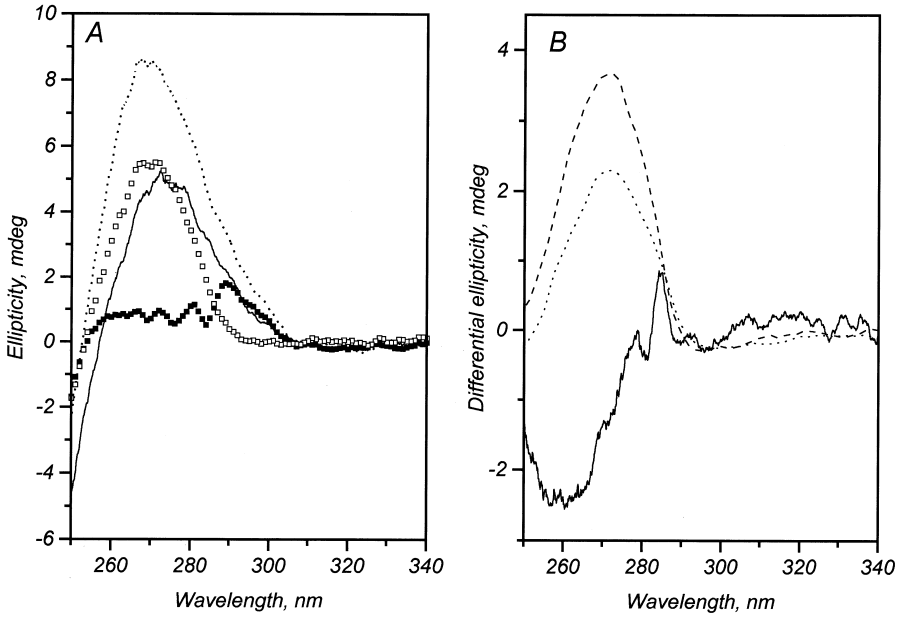


Fig. 2. Altered binding of α,β-imido-dUTP to native or truncated dUTPase as recorded by near UV CD. A: Spectra of native enzyme alone (closed symbols), α,β-imido-dUTP alone (open symbols) (scattered graphs), and the mixtures (line graphs) of α,β-imido-dUTP with native (solid line) and truncated (dotted line) dUTPase. The spectrum of truncated enzyme is identical to the spectrum of the native enzyme in this wavelength range [13]. B: Difference spectra of native dUTPase with α,β-imido-dUTP (solid line) or dUDP (dashed line). The dotted line is the difference spectrum of truncated dUTPase with α,β-imido-dUTP. Protein and ligand concentration were 45 μM and 60 μM, respectively.

argue for an involvement of Phe in the binding of the triphosphate analogue.

The difference signals proved to be saturable (Fig. 3) allowing determination of apparent dissociation constants, $K_{d,app}$ (Table 1). The result that the triphosphate analogue binds significantly (about 10-fold) more strongly to the enzyme than dUDP demonstrates the importance of the γ-phosphate moiety of the analogue in the interaction with the enzyme. Both far and near UV CD gave, within experimental error, the same $K_{d,app}$ for the α,β-imido-dUTP-dUTPase complex, corroborating that the CD signals describe the interaction adequately. No difference spectra could be recorded if either Mg^{2+} or the analogues were omitted from the mixtures (data not shown), consistent with an interaction of the Mg^{2+} complexes of the analogues with the enzyme.

3.2. Involvement of the C-terminus in the binding of α,β-imido-dUTP

Specific tryptic hydrolysis at Arg-141 in *E. coli* dUTPase removes the C-terminal 11 residues, comprising most of Motif 5 [13]. The truncated protein does not show any conformational change upon complexation with α,β-imido-dUTP as monitored by far UV CD (Fig. 1B, dotted line). Binding of the analogue can still be followed by near UV CD. However, the difference signal (Fig. 2B, dotted line) is very similar to the signal elicited in the dUDP-native dUTPase complex (Fig. 2B, dashed line, positive peak at 270 nm), while the α,β-imido-dUTP-native dUTPase complex is characterized by a negative ellipticity peak at 260 nm (Fig. 2B, solid line). The conclusion is that loss of the C-terminus precludes formation of the binding pattern characteristic for α,β-imido-dUTP and the triphosphate analogue-induced protein conformational change. The strength of complex formation between α,β-imido-dUTP and truncated dUTPase is in accordance with this conclusion: the apparent dissociation constant is 10 μM, close to that of the dUDP-dUTPase complex (Table 1).

Trypsinolysis of *E. coli* dUTPase leads to a rapid decrease in enzyme activity [13]. The presence of the triphosphate analogue α,β-imido-dUTP during trypsinolysis has a significant protective effect (Fig. 4). The apparent first order rate constant for the activity loss, probably reflecting the specific hydrolysis of the Arg-Gly peptide bond (residues 141–142), was estimated to 0.038 min^{-1} and 0.0082 min^{-1}, in the absence or presence of α,β-imido-dUTP, respectively. The Arg-Gly peptide bond is part of the flexible C-terminus and the protective effect exerted by the triphosphate analogue reinforces the conclusion that the C-terminus is involved in binding of α,β-imido-dUTP.

Table 1
Interaction of di- and triphosphate substrate analogues with dUTPase

dUTPase	Ligand	Technique	$K_{d,app}$ (μM)	Δ[Θ] (mdeg/μM/cm)
Native	α,β-imido-dUTP	near UV CD (260 nm)	1 ± 0.4	0.06 ± 0.01
		far UV CD (206 nm)	1.1 ± 0.5	4.2 ± 1.5
		kinetics [14]	5	–
	dUDP	near UV CD (270 nm) [12,13]	10–15	0.07–0.09
		kinetics [15]	15	–
Truncated	α,β-imido-dUTP	near UV CD (270 nm)	10 ± 3	0.08 ± 0.02
	dUDP	near UV CD (270 nm) [12,13]	15	0.07

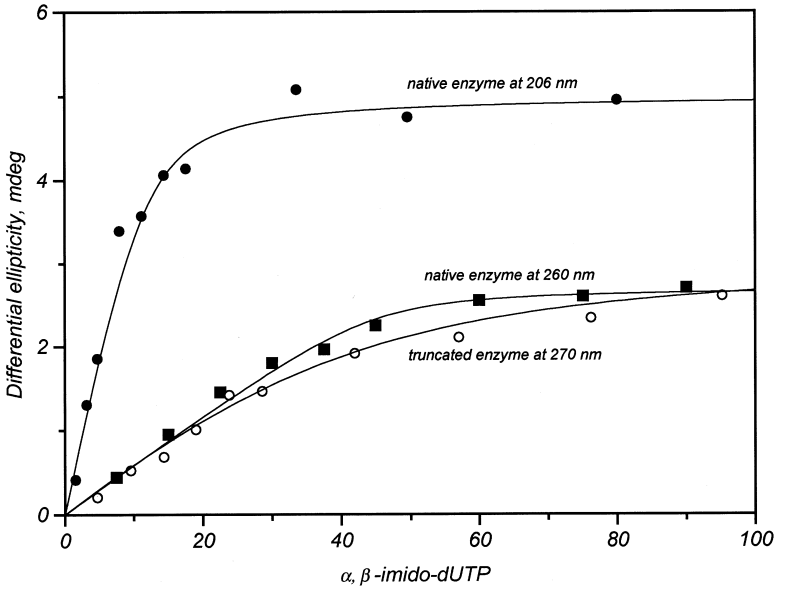


Fig. 3. Titration of enzyme with α,β-imido-dUTP. Native (closed symbols) or truncated (open symbols) dUTPase was titrated by stepwise addition of α,β-imido-dUTP. Absolute values of the differential CD signal at 206 nm (closed circles), 260 nm (closed squares) or 270 nm (open circles) are plotted. Data points were fitted (solid lines) to the equation: $\Delta\Theta/\Delta[\Theta] = (a-(a^2-4c_1c_2)^{0.5})/2$. $\Delta\Theta$ is the absolute value of the differential ellipticity determined by subtracting the ellipticity of α,β-imido-dUTP and dUTPase solution, measured separately, from the ellipticity measured in their mixture, $\Delta[\Theta]$ is the differential molar ellipticity of the protein-ligand complex, $a = c_1+c_2+K_{d,app}$, c_1 and c_2 are enzyme and ligand concentrations, respectively). $\Delta[\Theta]$ and $K_{d,app}$ (apparent dissociation constant) values are given in Table 1. Enzyme concentration for the far UV experiment (206 nm) was 12.5 μM, for the near UV experiments (260 or 270 nm) was 45 μM (native enzyme) or 40 μM (truncated dUTPase).

4. Discussion

The conserved Motif 5 at the flexible C-terminal arm of trimeric dUTPases cannot be located in the unliganded crystal structures of the enzyme from *E. coli*, *Homo sapiens* or the lentivirus of feline immunodeficiency [7,9,17]. Its functional role has been established for the protein from *E. coli* and the lentivirus of equine infectious anemia: loss of the motif or replacement of its conserved residues is deleterious for enzyme activity [13,18]. In the human enzyme, the C-terminal arm closes upon the active site when mono-, di- or triphosphates of 2′-deoxyuridine bind to the enzyme in the absence of Mg^{2+} [7]. However, no such movement can be seen when dUDP binds to the bacterial enzyme [10]. In the present study we identified a significant change in conformation of the bacterial enzyme induced exclusively in the presence of Mg^{2+} by the α,β-imido-triphosphate analogue of dUTP but not by dUDP. This conformational change, most probably, reflects

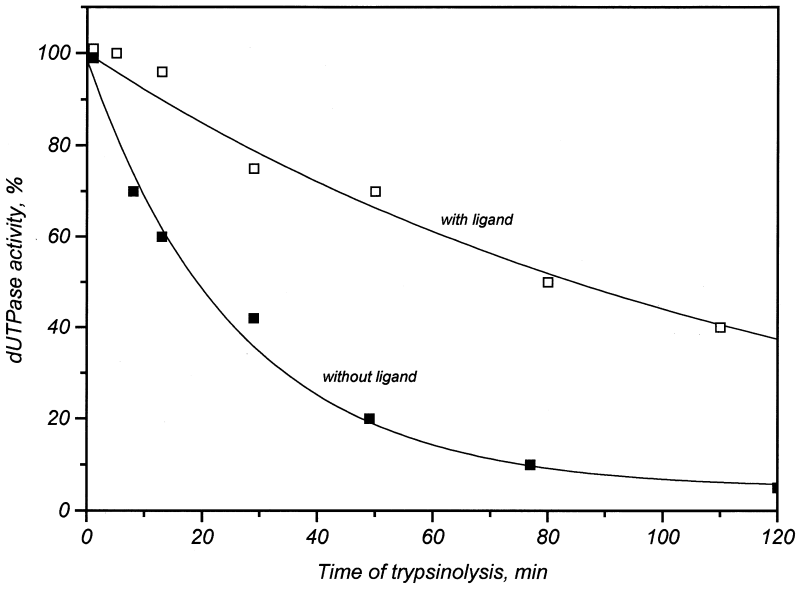
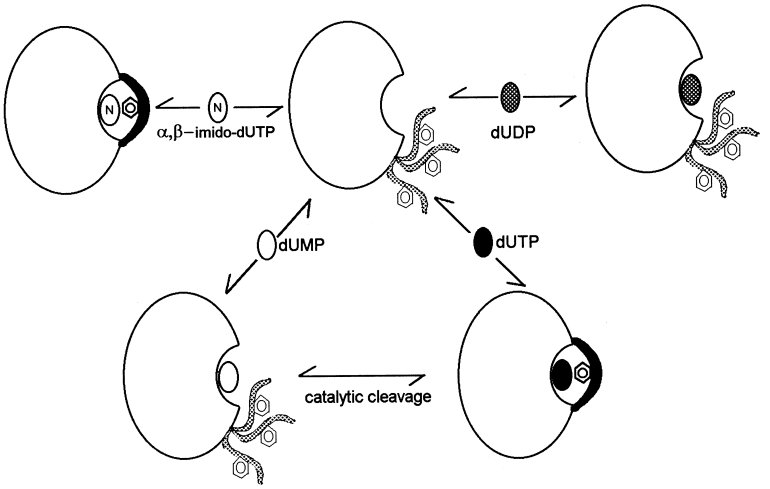


Fig. 4. α,β-Imido-dUTP protects against tryptic digestion at Arg-141. Native dUTPase (120 μM) was digested in the absence (closed symbols) or presence of ligand (200 μM, open symbols). Aliquots were taken from the reaction mixture at time intervals and assayed for enzyme activity. Data were fitted to first order reactions (solid lines).



Scheme 1. Molecular model for the catalytic cycle. The flexible C-terminus may adopt different conformations (light gray shades) in the unliganded, dUMP- or dUDP-containing protein. In the α,β-imido-dUTP and dUTP complexes, the tail becomes ordered (black shade). The benzene ring emphasizes the presumed role of Phe-146 in ligand accommodation. Shading of the C-terminus reflects the fact that this region is provided by a neighboring subunit in the trimeric dUTPase [7].

a movement of the C-terminal arm since removal of the arm abolishes the effect. The exact conformation of the C-terminal arm in the complex of the bacterial enzyme with α,β-imido-dUTP cannot be derived from our spectroscopic data. However, a piece of evidence suggests that this conformation is similar to the one seen in the human enzyme-ligand structures where the strictly conserved Phe of the C-terminus (part of Motif 5) stacks on top of the uracil ring [7]. The near UV CD difference signal specific for the α,β-imido-dUTP-enzyme complex has a negative peak at 260 nm which is the absorbance maximum of Phe and uridine. We therefore argue that this signal may be due to a Phe-uracil stacking, similarly to the structure of the human enzyme-ligand complex. The bacterial enzyme also possesses the conserved Phe residue, at position 146 in Motif 5 [13].

The apparent dissociation constants for the native enzyme-ligand complexes are in good agreement with the respective K_i values determined by kinetic measurements (Table 1), rendering support for rapid equilibrium steps in inhibitor binding and release. From the $K_{d,app}$ values for the complexes of α,β-imido-dUTP with native or truncated dUTPase, it can be estimated that a significant portion (about 20%) of the binding energy is due to the interaction provided by the C-terminal arm.

A possible molecular model for the catalytic cycle is presented in Scheme 1. The enzyme can be either in an open conformation with the C-terminal arm disordered, or in a closed state with the arm in a more ordered conformation and associated with the active site. The open/closed conformational shift induces the far UV difference CD signal and the near UV negative difference peak at 260 nm. Both the complete triphosphate moiety of the nucleotide in complex with Mg^{2+} and the C-terminal arm of the protein are indispensable for the closed conformation. The catalytic cycle is proposed to be initiated by the dUTP binding-induced formation of the closed conformer which allows cleavage of the α,β-phosphoanhydride bond. Upon cleavage the active site opens up and the products dUMP and PP_i can be released. α,β-Imido-dUTP is non-hydrolyzable probably because the nitrogen of the imido group, replacing the bridging oxygen at the scission position, is less electrophilic. Thereby, the partial positive charge on the α phosphorus atom is decreased making it less susceptible to a nucleophilic attack, as suggested for the non-hydrolyzable imido analogue of ATP [19]. The model in Scheme 1 provides a plausible explanation for the fact that dUDP is not hydrolyzed by the enzyme. This compound, which contains an α,β-phosphoanhydride bond as in dUTP, is not capable of inducing the closed conformation necessary for catalysis.

Mg^{2+} was found to be necessary for the conformational changes studied here, while the human enzyme-ligand complexes, having most of the flexible tail visible in the electron density maps, do not contain Mg^{2+} [7]. It is likely that the very high (millimolar) protein and ligand concentrations, used in the crystallization but out of reach in our spectroscopic experiments, render the fixation of the C-terminus possible even in the absence of Mg^{2+}. However, the Mg^{2+}-free complex structures should be expected to be catalytically non-productive, providing incomplete mechanistic information.

In conclusion, we provide evidence for a conformational change of the C-terminal flexible arm of *E. coli* dUTPase induced by α,β-imido-dUTP in complex with Mg^{2+}. The catalytic model, based on the experimental results, explains facilitated product release and the non-hydrolyzable character of dUDP.

References

[1] Bertani, L.E., Haggmark, A. and Reichard, P. (1963) J. Biol. Chem. 238, 3407–3413.
[2] Pearl, L.H. and Savva, R. (1996) Nature Struct. Biol. 3, 485–487.
[3] El-Hajj, H.H., Zhang, H. and Weiss, B. (1988) J. Bacteriol. 170, 1069–1075.
[4] Gadsden, M.H., McIntosh, E.M., Game, J.C., Wilson, P.J. and Haynes, R.H. (1993) EMBO J. 12, 4425–4431.
[5] Pyles, R.B., Sawtell, N.M. and Thompson, R.L. (1992) J. Virol. 66, 6706–6713.
[6] Threadgill, D.S., Steagall, W.K., Flaherty, M.T., Fuller, F.J., Perry, S.T., Rushlow, K.E., LeGrice, S.F.J. and Payne, S.L. (1993) J. Virol. 67, 2592–2600.

[7] Mol, C.D., Harris, J.M., McIntosh, E.M. and Tainer, J.A. (1996) Structure 4, 1077–1092.
[8] McGeoch, D.J. (1990) Nucleic Acids Res. 18, 4105–4110.
[9] Cedergren-Zeppezauer, E.S., Larsson, G., Nyman, P.O., Dauter, Z. and Wilson, K.S. (1992) Nature 355, 740–742.
[10] Larsson, G., Svensson, L.A. and Nyman, P.O. (1996) Nature Struct. Biol. 3, 532–538.
[11] Hoffmann, I., Widström, J., Zeppezauer, M. and Nyman, P.O. (1987) Eur. J. Biochem. 164, 45–51.
[12] Vertessy, B.G., Persson, R., Rosengren, A.M., Zeppezauer, M. and Nyman, P.O. (1996) Biochem. Biophys. Res. Commun. 219, 294–300.
[13] Vertessy, B.G. (1997) Proteins 28, 568–579.
[14] Persson, T., Larsson, G. and Nyman, P.O. (1996) Bioorg. Med. Chem. 4, 553–556.
[15] Larsson, G., Nyman, P.O. and Kvassman, J. (1996) J. Biol. Chem. 271, 24010–24016.
[16] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[17] Prasad, G.S., Stura, E.A., McRee, D.E., Laco, G.S., Hasselkus-Light, C., Elder, J.H. and Stout, C.D. (1996) Protein Sci. 5, 2429–2437.
[18] Shao, H., Robek, M.D., Threadgill, D.S., Mankowski, L.S., Cameron, C.E., Fuller, F.J. and Payne, S.L. (1997) Biochim. Biophys. Acta 1339, 181–191.
[19] Yount, R.G., Babcock, D., Ballantyne, W. and Ojala, D. (1971) Biochemistry 10, 2484–2489.