Human dUTPase is essential in controlling relative cellular levels of dTTP/dUTP, both of which can be incorporated into DNA. The nuclear isoform of the enzyme has been proposed as a promising novel target for anticancer chemotherapeutic strategies. The recently determined 3D structure of this protein in complex with an isosteric substrate analogue allowed in-depth structural characterization of the active site. However, fundamental steps of the dUTPase enzymatic cycle have not yet been revealed. This knowledge is indispensable for a functional understanding of the molecular mechanism and can also contribute to the design of potential antagonists. Here we present detailed pre-steady state and steady-state kinetic investigations using a single tryptophan fluorophore engineered into the active site of human dUTPase. This sensor allowed distinction of the apoenzyme, enzyme-substrate and enzyme-product complexes. We show that the dUTP hydrolysis cycle consists of at least four distinct enzymatic steps: i) fast substrate binding, ii) isomerization of the enzyme-substrate complex into the catalytically competent conformation, iii) hydrolysis (chemical) step iv) and rapid, non-ordered release of the products. Independent quench-flow experiments indicate that the chemical step is the rate-limiting step of the enzymatic cycle. To follow the reaction in the quench-flow, we devised a novel method to synthesize $^{32}\text{P}$-labelled dUTP. We also determined by indicator-based rapid kinetic assays that proton release is concomitant with the rate-limiting hydrolysis step. Our results led to a quantitative kinetic model of the human dUTPase catalytic cycle and to direct assessment of relative flexibilities of the C-terminal arm, critical for enzyme activity, in the enzyme-ligand complexes along the reaction pathway.

dUTPase is the unique enzyme that specifically hydrolyzes the $\alpha$-$\beta$ pyrophosphate bond of dUTP to yield dUMP and inorganic pyrophosphate (PPi) (1). The enzyme is essential in maintaining DNA integrity in dividing cells (2,3). Its activity is responsible for setting the physiological dUTP:dTTP concentration ratios (1:24, (4)), thus preventing high rates of uracil incorporation into newly synthesized DNA. Although uracil in DNA is tolerated to a certain level by the base excision DNA repair mechanisms (BER), higher levels of uracil in DNA trigger double-strand breaks and lead to cell death (5). Several lines of evidence show that upregulated dUTPase is responsible for desensitizing tumors to drugs inhibiting the thymidylate synthase pathway, thus acting as an important survival factor for tumor cells (6,7). Increased levels of the nuclear isoform of the enzyme correlate to worsened prognosis of several tumors, as revealed by detailed analysis of tissue samples (8,9). dUTPase has therefore emerged as a high-potential anticancer drug target, which possesses several additional, possibly advantageous features for drug design. Unlike most nucleotide metabolizing enzymes, dUTPase is extremely specific to its substrate nucleotide, potentially allowing construction of substrate analogue antagonists with similarly high specificity. The nuclear isoform of the enzyme is under strict cell-cycle control; its expression is mostly limited to rapidly dividing (including cancer) cells (10,11). In addition to the fact that the enzyme is an important focus in biomedical research, dUTPase also serves as a model system for detailed analysis of enzyme-catalyzed nucleotide pyrophosphorolysis.

Current knowledge of the dUTPase mechanism is mainly based on 3D structural approaches. Most dUTPases are homotrimers with a unique active site architecture where all three monomers contribute to each of the three catalytic sites. High resolution crystal structures of the human (hDUT)
(12,13) and other (14-18) dUTPases provided important mechanistic clues. The catalytic site is formed by five conserved motifs, four of which are contributed by two adjacent monomers. The fifth motif, positioned on the C-terminal arm, is usually provided by the third monomer. The C-terminus, associated with an increased conformational freedom, was suggested to close upon the active site during the chemical step (12,14,19). Cleavage of the α-β pyrophosphate linkage is initiated by a nucleophilic attack from the catalytic water molecule coordinated by a conserved aspartate (Asp102 in the human enzyme) within the third motif accommodating the uracil and deoxyribose moieties of dUTP (16).

Modern pharmacology demands knowledge of the precise mechanism of action of target enzymes. However, structural data have not yet been complemented by detailed solution kinetic studies for any eukaryotic dUTPase, possibly due to the lack of suitable optical signals reporting enzymatic events. Relying on proton escape during nucleotide pyrophosphorolysis, pH indicator-based assays were used to continuously follow dUTP hydrolysis (20,21), but these methods are transparent to conformational changes of the enzyme. In this study, we took advantage of an intrinsic tryptophan sensor that we had recently engineered in the C-terminal arm of hDUT (Trp158) (13) to resolve the fundamental steps of the enzymatic cycle using fast kinetic methods. Trp158 replaces a conserved phenylalanine residue that interacts with the uracil ring of dUTP (FIGURE 1C, (12)(13)). The mutational replacement of the benzene ring with an indole moiety did not perturb the enzyme activity (13). In the present study, the active site Trp158 sensor also allowed assessment of the proposed structural ordering of the C-terminal arm in the distinct enzyme-ligand complexes, relevant for the reaction cycle. Furthermore, we have developed a protocol for quench-flow analysis, which is the first to allow the direct monitoring of the hydrolysis step of a dUTPase. We unambiguously show that the chemical step is rate limiting and that the C-terminal arm is predominantly ordered in all enzymatic states.

**EXPERIMENTAL PROCEDURES**

*Materials* – The His-tagged nuclear isoform of human dUTPase (hDUT) and its Phe158Trp mutant construct (hDUT W158), were expressed and purified as described previously in (13,22). Protein concentration was measured using the Bio-Rad Protein Assay reagent and by UV absorbance ($\lambda_{280} = 10430 \text{ M}^{-1}\text{cm}^{-1}$ for hDUT and $\lambda_{280} = 15930 \text{ M}^{-1}\text{cm}^{-1}$ for hDUT W158) and is given in monomers. All measurements were carried out in 20 mM HEPES pH 7.4 buffer, also containing 40 mM NaCl, 2 mM MgCl2 and 1 mM DTT (unless otherwise stated), at 20 °C. dUMP, dUDP, dUTP and $\alpha$, $\beta$-iminodUTP (dUPNPP) were purchased from Jena Bioscience (Germany), $\gamma^{32}$P-ATP from Izinta Ltd. (Hungary). Myosin was purified from rabbit skeletal muscle according to (23). Other reagents were from Sigma-Aldrich.

Enzyme activity was measured in steady-state pH indicator-based assays as described in (20) and was typically found to be $6 \pm 2 \text{ s}^{-1}$. Active site titration was used to determine $K_M$ and also to evaluate the active fraction of hDUT and hDUT W158 preparations. In the absorbance stopped-flow setup, an assay buffer containing 100 µM phenol red indicator and 1 mM HEPES pH 7.5 provided optimal monitoring of dUTP hydrolysis. To avoid mixing artifacts, the enzyme was dialyzed in this assay buffer prior to active site titration. Measured time courses (cf. FIGURE 3C) were subjected to global fit analysis using GEPASI (24). The floated parameters were $k_1$, $k_{-1}$, $k_2$ and [E] of the Michaelis-Menten scheme:

$$\begin{align*}
E + S & \rightleftharpoons ES \\
& \rightarrow EP
\end{align*}$$

where $K_M = (k_{-1} + k_2) / k_1$ and $k_2 = k_{cat}$. The inactive protein fraction in the measured hDUT or hDUT W158 preparation was only in the range of the uncertainty of protein concentration determination (5-10 %).

Fluorescence spectra and intensity titrations were recorded on a Jobin Yvon Spex Fluoromax-3 spectrofluorometer with excitation at 297 nm (slit 1 nm), emission between 320-400 nm (slit 5 nm) or at 347 nm. Because large concentrations of nucleotides were used, care was taken to correct for any additional fluorescence or inner filter effect imposed on the measured intensities by the nucleotide stock solutions.

Acrylamide quenching was carried out by the addition of minute volumes of a 5 M acrylamide solution to the enzyme, enzyme-ligand or N-acetyl...
L-tryptophanamide (NATA) solutions. Raw data were corrected for the fluorescence arising from the acrylamide solution itself. \( F_0 / F \) vs. \([Q]\) curves were analyzed using a modified Stern-Volmer equation (Equation 1):

\[
F_0 / F = (1 + K_{SV} [Q] \exp (V[Q])) \quad \text{Equation 1}
\]

where \( F_0 \) is the unquenched, \( F \) is the quenched fluorescence; \( Q \) is the quencher; \( K_{SV} \) is the dynamic (bimolecular) quenching constant whereas \( V \) is the static (sphere of action) component of quenching (cf. (25)).

Fluorescence anisotropy was measured by the single-channel method in an Edinburgh Instruments FLS920P Spectrofluorometer equipped with Glan-Thompson prism polarizers. Tryptophan emission spectra (\( \lambda_{ex} = 295 \text{ nm}, \lambda_{em} = 320-400 \text{ nm} \)) were recorded at four different polarizer configurations (VV, VH, HV, HH; V and H denote vertical and horizontal polarizer configurations, respectively, the first letter being designated to the excitation, the second to the emission polarizer.). After baseline correction, anisotropy was calculated for the entire spectrum using the equation (Equation 2):

\[
r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}) \quad \text{Equation 2}
\]

where \( r \) is anisotropy, \( I \) is fluorescence intensity and \( G = I_{HV} / I_{HH} \) is a wavelength-dependent parameter of the instrument setup.

Stopped-flow experiments – Measurements were done using either a SF-2004 (KinTek Corp., Austin, TX) or a SFM-300 (Bio-Logic SAS, France) stopped-flow apparatus. Tryptophan fluorescence was excited at 297 nm and emission was selected with a band-pass filter having a peak in transmittance at 340 nm. Time courses were analyzed using the curve fitting software provided with the stopped-flow apparatus or by Origin 7.5 (OriginLab Corp., Northampton, MA).

\( \gamma^{32}\text{P}-\text{dUTP synthesis} \) – All synthesis reactions were carried out in a buffer containing 25 mM TRIS pH 7.4 and 100 mM NaCl. Autophosphorylation of 20 \( \mu \text{M} \) nucleoside diphosphate kinase (NDPK from yeast, Sigma N0379) was carried out in 5 mM EDTA at 30 °C for 10 minutes in a final volume of 100 \( \mu \text{l} \) using 20 \( \mu \text{M} \) \( \gamma^{32}\text{P}-\text{ATP} \), according to (26). To remove ADP and \( \gamma^{32}\text{P}-\text{ATP} \) from the reaction in a quick manner, we applied batch adsorption on anion exchanger resin in an expectation that the resin will only remove the negatively charged nucleotides, but will not bind NDPK. The NDPK isoenzyme used in the experiment has a calculated pi of 8.65 and therefore carries a net positive charge at pH 7.4. According to the expectation, 25 \( \mu \text{l} \) of washed Q-Sepharose (Amersham, GE) added to the autophosphorylation reaction mixture immobilized all nucleotides without binding NDPK. The Q-Sepharose beads were then removed from the reaction by a 30 second centrifugation step. Subsequently, 25 \( \mu \text{M} \) dUDP and 10 mM Mg\(^{2+}\) were added to \( \gamma^{32}\text{P}-\text{NDPK} \) to yield \( \gamma^{32}\text{P}-\text{dUTP} \) (incubation for 10 minutes at 30 °C). The enzyme was then completely removed from the \( \gamma^{32}\text{P}-\text{dUTP} \)-containing solution by phenol extraction that was carried out according to (27). For the analysis of the synthesis products (see RESULTS), the radioactive nucleotide and phosphate contents were separated from each other using charcoal adsorption (as in (28)). The advantage of using charcoal is that it binds all nucleotides but not Pi and PPI. Radioactivity was counted in water in a Wallac 1409 Liquid Scintillation Counter. We used a \( \gamma^{32}\text{P}-\text{ATP} \) stock solution of high specific activity (0.4 MBq/\( \mu \text{l} \), 111 GBq/\( \mu \text{mole} \)) to obtain a similarly high specific activity \( \gamma^{32}\text{P}-\text{dUTP} \) sample suitable for tracing. The synthesized \( \gamma^{32}\text{P}-\text{dUTP} \) was added to a large molar excess of non-labeled bulk dUTP in a 1:100 volume ratio. For further details of the analysis of the synthesis products see RESULTS.

Quench flow experiments were carried out using the RQF-3 (KinTek Corp., Austin, TX) quench-flow apparatus. 2 M HCl (2/3 M in the reaction) was used as the chemical quencher of the dUTPase reaction. Hydrolysis products were separated according to (28). The amount of the resulting \( \gamma^{32}\text{P}-\text{Pi} \) product was counted in water using a Wallac 1409 Liquid Scintillation Counter (PerkinElmer, Inc.).

Data analysis and numerical simulations were done using Origin 7.5 (OriginLab Corp., Northampton, MA) or the freely available GEPASI 3 Biochemical Kinetics Simulation software (24), respectively.

RESULTS

Fluorescence spectral properties of hDUT\(^{W158} \) and its ligand-bound complexes.
Recently, fluorescent emission from the Trp158 fluorophore was shown to be significantly and characteristically quenched in dUTPase-dUPNPP and dUTPase-dUMP complexes as compared to the apoenzyme (13), in agreement with the expectation that the stacking between conserved residue Phe158 and the substrate uracil ring (cf. FIGURE 1C) is also present in the Trp158 mutant enzyme. Following these observations, we quantified maximal fluorescence changes and spectral shifts of Trp158 upon binding to physiological ligands and to the non-hydrolysable substrate analogue dUPNPP (FIGURE 1A, TABLE 1). These data yield information on the interaction of Trp158 with the uracil moiety of any bound nucleotide, and will allow interpreting the fluorescence-based kinetic experiments. The Trp158 fluorescence emission maximum of the apoenzyme was at 353 nm, a typical value for a non-buried protein tryptophan (\( \lambda_{\text{max}} \)) (25) (FIGURE 1A). FIGURE 1A also shows that the binding of different uracil nucleotides but not that of PPi to hDUTW158 quenches Trp158 fluorescence, probably due to aromatic stacking between the indole and uracil rings (shortest distances between atoms of the uracil moiety and those of the Phe158 benzene ring in hDUT are 3.4-3.7 Å, as determined in the crystal structure of the enzyme-dUPNPP complex, PDB ID: 2HQU, (13), FIGURE 1C). The magnitude of the nucleotide-induced quench and blue shift increased in the order dUMP → dUDP → dUPNPP (TABLE 1). This implies that the presence of the β and γ phosphates causes the C-terminal arm to form more interactions with the phosphate chain of the substrate (in agreement with the structural description (13)), whereby the arm may become less flexible and may stabilize the stacking interaction between Trp158 and the uracil ring. Interestingly, Trp158 fluorescence was even more quenched during steady-state dUTPase cycling than in any of the other ligand-bound states (FIGURE 1A). This suggests that there is at least one major steady-state intermediate that cannot be produced by the addition of the above ligands (e.g. the pre-hydrolysis mimic dUPNPP or the post-hydrolysis mimic dUMP.PPi states). A possible explanation for this finding is that a particular protein conformational change occurs in the presence of dUTP (but not in the presence of dUPNPP or other nucleotides) leading to the hydrolysis-competent state (see below).

The large fluorescence increase in the presence of PPi indicates that the binding of this ligand also causes a conformational change in the active site. Control experiments conducted with bovine serum albumin and NATA (data not shown) ascertained that the effect of PPi on our tryptophan sensor was specific. Interestingly, a rather similar phenomenon was observed in an earlier study in which a tryptophan engineered into the entrance of the nucleotide binding site of myosin (W129, in close proximity to the adenine moiety of ATP) exhibited a large quench on nucleotide binding, and a large fluorescence increase on PPi binding (29). We probed the potential interaction of hDUTW158 with phosphate (Pi) (used at high excess) but no signal change was detected.

**Fluorescence intensity titrations to determine enzyme-ligand dissociation constants.** FIGURE 1B shows fluorescence intensity titrations of 4 µM hDUTW158 with various ligands (enzyme active sites concentrations are used throughout this paper). Dissociation constants are given in TABLE 1. \( K_d \) values illustrate that the affinity of hDUTW158 increases in the order dUMP → dUDP → dUPNPP, with dUDP and dUPNPP binding being three and ten times stronger than that of dUMP, respectively. dUTP binding cannot be measured using this equilibrium method but we anticipate that its \( K_d \) may be equal to or lower than that of dUPNPP (\( K_d \sim 1 \mu M \)). Nucleotide-free hDUTW158 exhibited a \( K_d \) for PPi of 146 µM. The dissociation constant of PPi for the ternary enzyme-products complex (E.dUMP.PPi) was about three times larger than that for E.PPi. This moderate antagonistic effect between the binding of dUMP and PPi to the enzyme is probably due to the repulsion between the negative charges of dUMP and PPi.

**Acrylamide quenching.** We have performed acrylamide quenching experiments with hDUTW158 to monitor the solvent accessibility of the Trp158 reporter (FIGURE 2A, TABLE 1). For reference and control, we also measured properties of NATA, a model compound for a rotationally free and maximally solvent accessible tryptophan residue. Acrylamide titrations of the Trp158 fluorescence intensity are displayed as Stern-
Volmer plots (FIGURE 2A inset) with a modified Stern-Volmer equation (Eq. 1) fitted to the data points to separate the dynamic component (described by the $K_{sv}$ quenching constant) from the static quenching sphere of action ($V$) (25). Compared to our measurement on NATA and literature data on $K_{sv}$ values for tryptophans in short peptides (10-14 M$^{-1}$), Trp158 exhibits markedly reduced solvent accessibility ($K_{sv} = 6.6$ M$^{-1}$) even in the apoenzyme. Such a low $K_{sv}$ was unexpected, considering that Trp158 is situated in the C-terminal arm of hDUT$^{W158}$, six residues away from the terminal amino acid. On the other hand, differences between $K_{sv}$ values of various ligand-bound states of hDUT$^{W158}$ are relatively small, but significant ($K_{sv}$ ranging from 5.0 to 6.8 M$^{-1}$) (FIGURE 2A, TABLE 1). This finding suggests that large conformational changes of the C-terminal arm upon ligand binding are unlikely to occur. The solvent accessibility of Trp158 decreases in the order dUMP $\rightarrow$ dUDP $\rightarrow$ dUPNPP, suggesting a gradual movement of the C-terminal arm towards the nucleotide. This observation is in line with our experiments shown in FIGURE 1. Importantly, the solvent accessibility of the E.dUMP.PPi and E.dUMP states were very similar, indicating that dUMP but not PPi induces shielding of the active site. PPi binding alone does not perturb the solvent accessibility of Trp158 probably due to a relatively open active site conformation (FIGURE 1).

**Fluorescence anisotropy.** Fluorescence anisotropy is routinely used to describe the dynamic properties of a protein environment. Freely rotating small fluorophores are depolarized at room temperature and therefore exhibit anisotropies close to zero (cf. NATA in FIGURE 2B and TABLE 1). We measured the steady-state anisotropies of apo-hDUT$^{W158}$ and its ligand-bound complexes to gain further insights into the dynamic behavior of the C-terminal arm in various enzymatic states. The steady-state anisotropy of apo-hDUT$^{W158}$ ($r = 0.077$) increased upon ligand binding, which reflects a steric hindrance of the fluorophore. Similarly to the previously described experiments (FIGURES 1 and 2A), a correlation of the measured effect to the length of the phosphate chain of the nucleotide was observed (i.e. the value of $r$ increased in the order apo $\rightarrow$ dUMP $\rightarrow$ dUDP $\rightarrow$ dUPNPP, FIGURE 2B). The largest increase in anisotropy was detected in the PPi-bound species (E.PPi and E.dUMP.PPi), even though we previously showed that these are the most “open” and solvent accessible enzyme states (FIGURE 2A). Taken together, the anisotropy data indicate that i) ligand binding to the polyphosphate binding site causes structural ordering of the C-terminal arm, proportionally to the length of the polyphosphate chain (without shielding Trp158 from the solvent); ii) the lower anisotropy of uracil nucleotide-bound states compared to that of the E.PPi state show that aromatic stacking to uracil slightly depolarizes Trp158 (FIGURE 2B, TABLE 1).

**Rapid kinetics of hDUT$^{W158}$ followed by intrinsic (Trp158) and extrinsic (proton release) signals.** In the knowledge of the fluorescence characteristics of individual enzyme-substrate (substrate analogue) and enzyme-products complexes (FIGURE 1), progress curves obtained by monitoring Trp158 fluorescence during the interaction of hDUT$^{W158}$ with dUTP in the stopped-flow yielded significantly more information than pH detection-based (proton release) methods. FIGURE 3 shows single and multiple dUTP turnovers obtained using Trp158 fluorescence (A-B) or proton release (C) signals. Trp158 fluorescence traces of single dUTP turnovers ([E] $> [S]$) consisted of three exponential phases (FIGURE 3A). A fast initial quench in fluorescence ($k_{obs} \approx 900$ s$^{-1}$) was followed by an additional slower decrease ($k_{obs} \approx 20$ s$^{-1}$), then the fluorescence intensity returned to a close-to-initial value with a $k_{obs}$ of $6.8 \pm 2.0$ s$^{-1}$. In the light of the steady-state fluorescence data of FIGURE 1A, we interpret the first fast phase as the initial binding of the nucleotide in which Trp158 quenching occurs by stacking over the uracil ring. Considering the difference between the fluorescence intensity of the enzyme-dUPNPP complex and that during steady-state dUTPase cycling, the second slower phase can be interpreted as a dUTP-induced structural change that precedes or is concomitant with dUTP hydrolysis. The third phase reflects the slowest rate-limiting step of the cycle (dUTP hydrolysis or product release). (The identities of the steps associated with the second and third phases were clarified in subsequent experiments (FIGURES 4-5)). Determination of the substrate concentration dependence of the rate constant of
the first phase under pseudo first order conditions was challenging because the signal to noise ratio was too low for reasonable resolution (when attempting to decrease \([S]\) at a constant \([E]\) (maintaining \([S] << [E]\)), or the amplitude became completely lost in the dead-time of the stopped-flow apparatus (when applying a several-fold excess of \([S]\) over the lowest detectable \([E]\)). Measurements carried out using near-equimolar concentrations of enzyme and substrate indicated that the time course of this phase does depend on concentration \((k_{\text{obs}} \text{ values of force-fitted exponentials were } 400-1200 \text{ s}^{-1} \text{ in the applied } 2.5-15 \mu M \text{ concentration range})\). Numerical simulations in which this phase was assigned to a second-order binding step showed good agreement with the experimental traces and the fundamental rate constants could be extracted (cf. FIGURE 6A, TABLE 2, below). We did not observe systematic concentration dependence of the \(k_{\text{obs}}\) (termed \(k_{\text{ISO}}\) in TABLE 2) of the second exponential phase \((20 \pm 18 \text{ s}^{-1})\), which confirms the first order nature of this proposed isomerisation step (FIGURE 3B). The \(k_{\text{obs}}\) of the third phase did not exhibit concentration dependence in the single turnover concentration regime. The \(k_{\text{obs}}\) of this phase was in good agreement with the previously determined steady-state \(k_{\text{cat}}\) of hDUT \((8 \pm 3 \text{ s}^{-1})\) (13), indicating that it represents the rate-limiting step of the dUTPase cycle. Furthermore, the duration of the steady-state \((t_{\text{ss}})\) in multiple turnover Trp158 fluorescence traces (i.e. the time elapsed between the start of the reaction and the inflection point of the fluorescence restoration phase, FIGURE 3B) was consistent with the above third phase \(k_{\text{obs}}\) and steady-state \(k_{\text{cat}}\) values \((t_{\text{ss}} \approx [S]_{\text{initial}}/([E]_{\text{total}}k_{\text{cat}}),\) if \([S]_{\text{initial}} \gg K_M\).

FIGURE 3C shows single and multiple turnovers detected by a proton release assay in an absorbance stopped-flow setup. The amplitude of the curves was directly proportional to the initial substrate (and thus the released proton) concentration. In single turnover conditions \((([E] > [S]),\) lower two curves in FIGURE 3C), the time courses corresponded to single exponentials, and \(k_{\text{obs}}\) values \((6.5 \pm 0.1 \text{ s}^{-1})\) were identical to the steady-state \(k_{\text{cat}}\) of the enzyme (TABLE 2). In multiple turnovers (upper two curves in FIGURE 3C) a linear steady-state phase was observed without any burst of proton release. These profiles altogether imply that the enzymatic cycle is limited by a single rate-limiting step that occurs before or is concomitant with proton release. We could model these proton release events with Michaelis-Menten kinetics in which a rapid equilibrium \((k_1, k_{-1})\) precedes the rate-limiting step \((k_2 = k_{\text{cat}}).\) Global fits to the single and multiple turnover time courses using the \(k_1, k_{-1}\) and \(k_2\) floating parameters of SCHEME 1 yielded \(K_M = 3.6 \pm 1.9 \mu M, k_{\text{cat}} = 6.7 \pm 0.2 \text{ s}^{-1}, k_{\text{cat}}/K_M \approx 1.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}\), for both hDUT and hDUTW158 proteins.

\(\gamma^{32}\text{P-dUTP synthesis.}\) \(\gamma^{32}\text{P-dUTP}\) is not commercially available. We therefore developed a straightforward synthesis method (FIGURE 4A) using nucleoside diphosphate kinase (NDPK) that converts \(\gamma^{32}\text{P-ATP}\) and dUDP into \(\gamma^{32}\text{P-dUTP}\) and ADP by a ping-pong mechanism (26,30). We took advantage of the fact that the phosphorylated enzyme intermediate of the NDPK reaction is long-lived in the absence of Mg2+ and thus can be separated from the phosphate donor nucleotides (26). The resulting synthesis product (after step 4 in FIGURE 4A) contains \(\gamma^{32}\text{P-dUTP},\) dUDP and inorganic phosphate. To test for the presence of any non-dUTP-derived radiolabeled species that would compromise radiochemical purity, aliquots of the synthesis product were fully hydrolyzed by \(i)\) dUTPase (extremely specific for dUTP), \(ii)\) dUTPase + myosin (hydrolyzes NTPs (31)) or \(iii)\) apyrase (hydrolyzes (d)NTPs and (d)NDPs (32)). All three enzyme conditions resulted in liberation of the same \(32\text{Pi}\) content of the total radioactive material, demonstrating that practically all hydrolysable radioactive nucleotide species in the synthesis product was \(\gamma^{32}\text{P-dUTP.}\) The synthesis product contained \((15 \pm 3)\%\) non-nucleotide \(32\text{Pi}\) (measured in samples from which all nucleotides had been removed). Analysis showed that this fraction originated from \(i)\) carryover from the original \(\gamma^{32}\text{P-ATP}\) solution \((5\%),\) \(ii)\) spontaneous hydrolysis of \(\gamma^{32}\text{P-nucleotides}\) during the 4-step procedure and possibly from \(iii)\) slow \(32\text{Pi}\) release from the phosphorylated NDPK in the absence of phosphate acceptor (during step 2). The ~15% \(32\text{Pi}\) in the synthesis product does not reflect the Pi content of the bulk solution to be used in quench-flow experiments, because a subsequent large dilution of the synthesis product in non-labeled dUTP decreased the Pi: dUTP concentration ratio to less than 1:10,000 in the reagent solution used in
the quench-flow assay. The only noticeable effect of the condition that ~15% of the total radioactivity is radioactive \(^{32}\)P is the reduction of the maximal expected signal change from 100% to 85%, which does not impede the evaluation of quench-flow results. Similarly, the dUDP contentration of the synthesis product was drastically reduced by the dilution of \(^{32}\)P-dUTP in a large molar excess of non-labeled dUTP (the dUDP:dUTP molar ratio was less than 1:1600 in the assay reagent). In the above described experimental conditions, the most important factor in providing chemical purity is the use of high quality non-labeled nucleotide to be traced with a high specific activity radioactively labeled one. The total \(^{32}\)P-dUTP yield was calculated following the analysis of the radioactive constitution of the synthesis product and was found to be 25 % (i.e. \(\frac{1}{4}\) of the \(^{32}\)P-ATP was converted specifically into \(^{32}\)P-dUTP). Considering that both reactions 1 and 3 (FIGURE 4A) are fully reversible, this yield indicates that the procedure was highly efficient.

**Direct observation of the chemical step by quench-flow using \(^{32}\)P-dUTP.**

FIGURE 4B shows a single turnover experiment with a single exponential fit to the data points. For both wild-type hDUT and hDUT\(^{W158}\) constructs and depending on the protein preparation, the \(k_{\text{H}}\) of single turnovers was determined to be \(5.5 \pm 2.5 \text{ s}^{-1}\), in agreement with the \(k_{\text{obs}}\) values observed in the fluorescent and proton release turnovers (TABLE 2). There was no systematic difference between the \(k_{\text{H}}\) values of hDUT and hDUT\(^{W158}\). When excess dUTP was mixed with hDUT (FIGURE 4C) we observed a linear steady-state phase without any burst, clearly arguing that the rate limiting step of the dUTPase enzymatic cycle is identical to (or precedes) the chemical step.

**Product release.** The large fluorescence intensity change of Trp158 induced by PPi binding allowed us to follow the dissociation of PPi from the enzyme. We carried out dUTP chase experiments to avoid rebinding of the dissociated PPi. Upon mixing the E.PPi complex with excess dUTP in the stopped-flow, double exponential curves were recorded (FIGURE 5, upper black trace) having a fast phase of \(740 \pm 66 \text{ s}^{-1}\), and a slow phase of \(24 \pm 6 \text{ s}^{-1}\). The amplitude of the fast but not the second slow phase depended upon the concentration of PPi (FIGURE 5 inset). The first phase can therefore be attributed to PPi dissociation whereas the second phase arises from the isomerisation of the ES complex occurring after the initial dUTP binding step (cf. FIGURE 3A). The \(K_d\) resulting from a one-binding-site quadratic fit to the amplitude data (\(327 \pm 117 \mu\text{M}\)) was similar to that obtained from equilibrium titrations (cf. FIGURE 1B, TABLE 1). Product dissociation was measured also from the E.dUMP.PPi complex (FIGURE 5, gray trace). Curves exhibited similar \(k_{\text{obs}}\) values (\(684 \pm 84 \text{ s}^{-1}\)) to the ones of the E.PPi curves, showing that the rate constants of PPi dissociation from E.PPi and E.dUMP.PPi are similar (TABLE 2). In line with the lower initial fluorescence level of E.dUMP.PPi compared to that of E.PPi (cf. FIGURE 1A), the amplitudes of the E.dUMP.PPi chasing traces were lower than those of the E.PPi chasing traces (FIGURE 5). Binding and dissociation of dUMP was too fast to observe by stopped-flow.

**Kinetic modeling of the hDUT enzymatic cycle.** The measured accessible parameters of the hDUT enzymatic cycle (TABLE 1-2) allowed us to propose a model that provided good fits to our experimental data (FIGURE 6B). Using this model, kinetic simulations of the hDUT\(^{W158}\) fluorescence profile during dUTP hydrolysis yielded time courses that were very similar to the measured ones (FIGURE 6A). Parameters for the binding (\(k_B, k_B^{-1}\), isomerization (\(k_{\text{ISO}}, k_{-\text{ISO}}\)) and hydrolysis (\(k_{\text{H}}\)) steps were floating parameters, given that these are the events that primarily determine the fluorescence profiles during dUTP turnovers. Kinetic parameters of the product release steps were fixed so that the ratios of the dissociation and association rate constants of the individual steps yield the \(K_d\) values shown in TABLE 1 and thus determine the final fluorescence levels. The rate constants of product release are so fast compared to the rate-limiting step that they do not influence the turnover curves.

**DISCUSSION**

A central aspect of the present study is that the fluorescent signal of a single tryptophan engineered into the C-terminal arm of hDUT (Trp158), which forms part of the active site, allowed precise resolution and characterization of
practically all key enzymatic steps. These steps include i) a rapid, probably diffusion limited substrate binding, ii) a subsequent substrate-induced structural change (isomerization) required for the formation of the catalytically competent conformation, iii) the rate limiting hydrolysis step and iv) rapid, non-ordered release of the hydrolysis products (FIGURE 6B, TABLE 2). The second isomerization step was not foreseen or suggested earlier due to the lack of conformationally sensitive assays to follow the reaction. Importantly, in the present work, two independent lines of evidence argue in favor of the existence of this isomerization step. First, the different extent of quenching and blue-shift associated with the enzyme-dUPNPP and enzyme-dUTP (steady-state) complexes (cf. FIGURE 1A) indicate the existence of at least two distinct pre-hydrolysis conformations of the active site. Second, the kinetic analysis of time courses in FIGURE 3A-B and FIGURE 6 clearly shows the presence of a slower exponential component following the initial fast binding of dUTP. An intriguing feature of the mechanism is that two different dUTP-bound intermediates will be significantly populated during steady-state dUTP hydrolysis (E.dUTP$^{††}$ will be predominant, but about 30% of the enzyme molecules will populate E.dUTP$^{†}$). This steady-state distribution results from the $k_{ISO}$ rate constant being in the same order of magnitude as the rate-limiting hydrolysis rate constant ($k_{H}$, FIGURE 6B).

We confirmed the rate limiting nature of the chemical (hydrolysis) step by $\gamma$$^{32}$P-dUTP-based quench-flow transient kinetic analysis (FIGURE 4). To obtain the commercially non-available $\gamma$$^{32}$P-dUTP, we developed a simple synthesis method based on the ping-pong phosphate transfer mechanism of NDPK (26). The novelty in our synthesis is that isolation of the $32$P-NDPK intermediate and the final $\gamma$-P-nucleotide product takes place in an Eppendorf tube, requires no instrumentation and results in a radiochemical purity that is suitable for many applications. Laborious purification of the synthesis products is not necessary because the donor and acceptor nucleotides are spatially and temporally separated. This straightforward method may be of great help in studying enzymes that use pyrimidine-triphosphates as substrate (e.g. dCTP deaminase, dTTPase, tRNA cytidyltransferase etc.), since none of these relevant $\gamma$-labeled pyrimidine nucleotides are commercially available (or may be purchased only as expensive custom synthesis orders). Due to the substrate promiscuity of NDPK, even base-modified nucleotide analogs may be radioactively labeled using this method for additional specific applications.

Interestingly, the estimated intracellular dUTP concentration ($\sim 0.7 \mu$M, (4)) is in the same range as the $K_M$ of hDUT for dUTP (TABLE 1-2). This indicates that dUTPase function is highly sensitive to cellular dUTP fluctuations around the physiological level. Our data show that product inhibition by dUMP at its estimated physiological concentration ($\sim 2.7 \mu$M, (4)) is probably not significant due to its relatively low affinity for hDUT and its rapid release from the enzyme-products complex (TABLE 1-2).

We probed in silico Phe158Trp mutations in available structures and found that the replacement of Phe158 with a Trp residue does not cause a steric hindrance within the active site. Accordingly, we found that hDUT$^{W158}$ retains the enzymatic activity of the wild-type enzyme. The presence of an aromatic residue at this location has been suggested to be important for enzyme activity (12), and thus the fact that a Trp residue can functionally replace the native Phe158 implies that the fluorescence signal reports events that are highly relevant to the physiological activity of the enzyme.

Trp158 is sensitive to the precise nucleotide (or other ligand) content of the active site (FIGURE 1A). The solvent shielding of Trp158 increases, while the structural flexibility of this residue decreases with increasing length of the polyphosphate chain of the nucleotide ligand (FIGURE 2). PPI binding into the binding site, however, causes a structural ordering of the arm without a solvent shielding effect. Based on our observation that E.PPI exhibits elevated fluorescence compared to the apoenzyme (FIGURE 1A), we speculate that PPI binding may cause disruption of a quenching interaction of Trp158, supposed to be present in the apo state. A possibility for such a quenching interaction is a cation-π type interaction (33) between Trp158 and the positively charged guanidino moiety of an arginine residing in its close proximity. Candidate arginines are residues 85, 128 and 135, all contributing to the binding and stabilization of the...
polyphosphate chain of the substrate nucleotide (FIGURE 7) (13). These groups are separated by about 9-10 Å from Trp158 via the intercalation of the uracil group in the hDUT-dUPNPP structure. In the apo state, however, one of the candidate arginines might move closer to the phenylalanine (tryptophan) to neutralize the positive charge via cation-π stacking, hence the intermediate fluorescence level observed in the apo-hDUT<sup>W158</sup>.

It is noteworthy that, while the binding of dUMP, dUDP and dUPNPP (and even more that of dUTP) to the enzyme causes marked quenching of Trp158 as compared to the apo state, the post-hydrolysis E.dUMP.PPi complex has an enhanced Trp158 fluorescence. We surmise that this fluorescence increase reflects a structural state in which the stacking interaction between Trp158 and the uracil moiety is at least partially disrupted, aiding the rapid release of products from this post-hydrolytic complex.

In addition to its utility in the determination of the kinetic and thermodynamic parameters, the Trp158 signal also provided much information about the structural dynamics of the C-terminal arm during catalysis. Evidence is presented that this protein segment is at least partially closed upon the active site in all enzymatic states (even including the apoenzyme) (FIGURE 2), and therefore its conformational freedom may be well restricted. FIGURE 7 shows that the C-terminal arm of monomer A is anchored in a β-sheet with the N-terminal residues of monomer C. At its very C-terminus, the arm also interacts with monomer B via strong H-bonds (second anchor). Interactions of the arm with the ligand dUPNPP are mainly formed by residues situated between the monomer-monomer interacting regions. This arrangement rationalizes i) the proposed proximity of the C-terminus to the protein core even in the absence of nucleotide (in the apoenzyme), and ii) that the C-terminal arm still conveys a significant flexibility in the apoenzyme (between the two anchor regions), as suggested in previous studies (12,13).

We described a complex methodology to assess the fundamental steps of dUTP hydrolysis as catalyzed by human dUTPase, an important chemotherapeutic target protein. The resulting novel insights underline the importance of the dynamic behavior of the C-terminal arm during catalysis and advocate the targeting of this enzyme segment for perturbation of dUTPase function.
REFERENCES

1. Shlomai, J., and Kornberg, A. (1978) *J Biol Chem* **253**(9), 3305-3312
2. Gadsden, M. H., McIntosh, E. M., Game, J. C., Wilson, P. J., and Haynes, R. H. (1993) *Embo J* **12**(11), 4425-4431
3. el-Hajj, H. H., Zhang, H., and Weiss, B. (1988) *J Bacteriol* **170**(3), 1069-1075
4. Traut, T. W. (1994) *Mol Cell Biochem* **140**(1), 1-22
5. Pearl, L. H., and Savva, R. (1996) *Nat Struct Biol* **3**(6), 485-487
6. Pugacheva, E. N., Ivanov, A. V., Kravchenko, J. E., Kopnin, B. P., Levine, A. J., and Chumakov, P. M. (2002) *Oncogene* **21**(30), 4595-4600
7. Chano, T., Mori, K., Scotlandi, K., Benini, S., Lapucci, C., Manara, M. C., Serra, M., Picci, P., Okabe, H., and Baldini, N. (2004) *Oncol Rep* **11**(6), 1257-1263
8. Ladner, R. D., Lynch, F. J., Groschen, S., Xiong, Y. P., Sherrod, A., Caradonna, S. J., Stoehlmacher, J., and Lenz, H. J. (2000) *Cancer Res* **60**(13), 3493-3503
9. Romeike, B. F., Bockeler, A., Kremmer, E., Sommer, P., Krick, C., and Grassner, F. (2005) *Pathol Res Pract* **201**(11), 727-732
10. Strahler, J. R., Zhu, X. X., Hora, N., Wang, Y. K., Andrews, P. C., Roseman, N. A., Neel, J. V., Turka, L., and Hanash, S. M. (1993) *Proc Natl Acad Sci U S A* **90**(11), 4991-4995
11. Bekesi, A., Zagyva, I., Hunyadi-Gulyas, E., Pongracz, V., Kovari, J., Nagy, A. O., Erdei, A., Medzhiradszky, K. F., and Vertessy, B. G. (2004) *J Biol Chem* **279**(21), 22362-22370
12. Mol, C. D., Harris, J. M., McIntosh, E. M., and Tainer, J. A. (1996) *Structure* **4**(9), 1077-1092
13. Varga, B., Barabás, O., Kovári, J., Tóth, J., Hunyadi-Gulyás, É., Klement, É., Medzhiradszky, K. F., Tölgyesi, F., Fidy, J., and Vertessy, B. G. (2007) *FEBS Lett, in press*
14. Larsson, G., Svensson, L. A., and Nyman, P. O. (1996) *Nat Struct Biol* **3**(6), 532-538
15. Prasad, G. S., Stura, E. A., Elder, J. H., and Stout, C. D. (2000) *Acta Crystallogr D Biol Crystallogr* **56**(Pt 9), 1100-1109.
16. Barabás, O., Pongracz, V., Kovari, J., Wilmanns, M., and Vertessy, B. G. (2004) *J Biol Chem* **279**(41), 42907-42915
17. Nemeth-Pongracz, V., Barabás, O., Fuxreiter, M., Simon, I., Pichova, I., Rumlova, M., Zabranska, H., Svergun, D., Petoukhov, M., Harmat, V., Klement, E., Hunyadi-Gulyás, E., Medzhiradszky, K. F., Konya, E., and Vertessy, B. G. (2007) *Nucleic Acids Res* **35**(2), 495-505
18. Dauter, Z., Persson, R., Rosengren, A. M., Nyman, P. O., Wilson, K. S., and Cedergren-Zeppezauer, E. S. (1999) *J Mol Biol* **285**(2), 655-673
19. Vertessy, B. G., Larsson, G., Persson, T., Bergman, A. C., Persson, R., and Nyman, P. O. (1998) *FEBS Lett*. **421**(1), 83-88
20. Vertessy, B. G. (1997) *Proteins* **28**(4), 568-579
21. Larsson, G., Nyman, P. O., and Kvassman, J. O. (1996) *J Biol Chem* **271**(39), 24010-24016
22. Kovári, J., Barabás, O., Varga, B., Békési, A., Tölgyesi, F., Fidy, J., Nagy, J., and Vertessy, B. G. (2007) *Proteins in press*
23. Margossian, S. S., and Lowey, S. (1982) *Methods Enzymol* **85 Pt B**, 55-71
24. Mendes, P. (1997) *Trends Biochem Sci* **22**(9), 361-363
25. Lakowicz, J. (1999) *Principles of fluorescence spectroscopy*, 2nd Ed., Springer
26. Munoz-Dorado, J., Inouye, S., and Inouye, M. (1990) *J Biol Chem* **265**(5), 2707-2712
27. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York
28. Li, X. D., Rhodes, T. E., Ikebe, R., Kambara, T., White, H. D., and Ikebe, M. (1998) *J Biol Chem* **273**(42), 27404-27411
29. Kovacs, M., Malnasi-Csizmadia, A., Woolley, R. J., and Bagshaw, C. R. (2002) *J Biol Chem* **277**(32), 28459-28467
30. Lascu, I., and Gonin, P. (2000) *J Bioenerg Biomembr* **32**(3), 237-246
31. White, H. D., Belknap, B., and Webb, M. R. (1997) *Biochemistry* **36**(39), 11828-11836
32. Molnar, J., and Lorand, L. (1961) *Arch Biochem Biophys* **93**, 353-363
33. Gallivan, J. P., and Dougherty, D. A. (1999) *Proc Natl Acad Sci U S A* **96**(17), 9459-9464

ACKNOWLEDGMENT

This work was supported by grants from Hungarian Scientific Research Fund (K68229), Howard Hughes Medical Institutes (#55005628 and #55000342), EMBO Long-Term Postdoctoral Fellowship to J. T., NIH Research Grant #D43 TW006230 (1 R01 TW007241-01) funded by the Fogarty International Center and the National Heart, Lung and Blood Institute, an EMBO-HHMI Startup Grant, and the Bolyai Fellowship of the Hungarian Academy of Sciences to MK; Alexander von Humboldt Foundation, Varga József Foundation, Hungarian Economic Competitiveness Operative Programme GVOP-3.2.1.-2004-05-0412/3.0, FP6 STREP 012127 and FP6 SPINE2c LSHG-CT-2006-031220. Thanks are due to Dr. Orsolya Barabás for useful comments on the manuscript and to the Department of Immunology of Eötvös Loránd University, Budapest for use of scintillation counter equipment.

ABBREVIATIONS

PPI, inorganic pyrophosphate; dUPNPP, α,β-iminodUTP; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; hDUT, nuclear isoform of human dUTPase, His-tagged; hDUTW158, Phe158Trp mutant of nuclear isoform of human dUTPase, His-tagged; NATA, N-acetyl L-tryptophanamide; NDPK, nucleoside triphosphate kinase; Bq, becquerel

FIGURE LEGENDS

FIGURE 1. (A) Fluorescence emission spectra of hDUTW158 at saturating concentrations of ligands. \( \lambda_{ex} = 295 \text{ nm} \), data are normalized to the emission peak of the apo enzyme. \([\text{hDUTW158}] = 4 \text{ \mu M} \), \([\text{dUMP}] = 500 \text{ \mu M} \), \([\text{dUDP}] = 300 \text{ \mu M} \), \([\text{dUPNPP}] = 100 \text{ \mu M} \), \([\text{dUTP}] = 2 \text{ mM} \), \([\text{PPi}] = 5 \text{ mM} \). To capture the dUTP-bound cycling steady-state, a high excess of dUTP was used and the spectrum was recorded within 30 seconds after dUTP was added. For analysis of fluorescence spectral parameters, see Table 1. (B) Fluorescence equilibrium titration of hDUTW158 with its ligands. \( \lambda_{ex} = 295 \text{ nm} \), \( \lambda_{em} = 347 \text{ nm} \), solid lines represent quadratic fits to the data except for the dUMP.PPi curve where Hill equation with an \( n = 1.7 \) provided a better fit. \( K_d \) values from the presented fits are: \( 31 \pm 4 \text{ \mu M} \) for dUMP (triangles), \( 12 \pm 2 \text{ \mu M} \) for dUDP (circles), \( 1.9 \pm 0.2 \text{ \mu M} \) for dUPNPP (diamonds), inset: \( 146 \pm 15 \text{ \mu M} \) for PPi (stars), \( 494 \pm 25 \text{ \mu M} \) for dUMP.PPi (crosses). The dUMP.PPi titration was carried out by titrating dUMP-saturated enzyme (in 500 \text{ \mu M} dUMP) with PPi. (C) Three-dimensional structure of hDUT in complex with dUPNPP (figure produced using PDB ID: 2HQU (13) and PyMOL). The three monomers (A, B, C) are represented by color-coded cartoons. One of the three active sites is shown with the bound dUPNPP (stick model, yellow carbons, otherwise atomic coloring). The Phe158 residue (monomer C) stacks over the uracil ring. Other coordinating residues from monomers A and B and the catalytic water molecule (red sphere, labeled \( W_{cat} \)) are shown for orientation purposes.

FIGURE 2. Solvent accessibility and anisotropy of hDUTW158 complexed with various ligands. (A) 4 \text{ \mu M} hDUTW158 with or without saturating concentrations of specific ligands was titrated using a 5 M acrylamide stock solution (inset). Lines on the data points are fits to the modified Stern-Volmer equation (Equation 1). The dynamic quenching components (\( K_q \) values) of the fits are shown as bars. \( 1 \text{ \mu M} \) NATA was used to represent a fully accessible tryptophan. Errors bars represent fitting errors. (B) Steady-state anisotropy of Trp158. Concentrations are the same as in FIGURE 1A. Error bars represent the S.D. of the data points obtained for each emission wavelength. 1 \text{ \mu M} NATA was used to represent a tryptophan exhibiting maximal rotational diffusion.
FIGURE 3. hDUTW158 single turnovers as monitored using intrinsic (A–B) and extrinsic (C) signals. (A) Tryptophan fluorescence stopped-flow traces traces of 7.5 µM hDUTW158 mixed with 5.25 µM dUTP (black points) or with buffer (grey points). Fluorescence was recorded at λex = 295 nm and λem = 340 nm. Solid line represents a triple exponential fit to the data with parameters A1 = 0.097, k1 = 912 s⁻¹ for dUTP binding, A2 = 0.193, k2 = 14 s⁻¹ for a first order isomerization, A1 = -0.269, k2 = 6.7 s⁻¹ for the chemical step. (B) Tryptophan fluorescence stopped-flow traces of 5 µM hDUTW158 mixed with various concentrations of dUTP (C) 58 µM hDUTW158 mixed with 25, 37.5, 50 or 75 µM dUTP in the stopped-flow in the presence of 100 µM phenol red indicator. Absorbance was recorded at λ = 559 nm to monitor the release of protons upon dUTP hydrolysis. Curves at substoichiometric dUTP concentrations appear as single exponentials whereas at higher dUTP concentrations a linear steady-state phase can be observed. Global fits to all curves using the k1, k1 and k2 floating parameters of the Michaelis-Menten scheme (SCHEME 1) yielded KM = 3.6 ± 1.9 µM, kcat = 6.7 ± 0.2 s⁻¹.

FIGURE 4. γ32P-dUTP synthesis and quench-flow measurements of dUTP hydrolysis. (A) Scheme of γ32P-dUTP synthesis. Reactions 1 and 3 are highly reversible while separation steps 2 and 4 are very efficient resulting in a γ32P-dUTP compound that is free of contaminating radioactive nucleotides. Besides γ32P-dUTP, the final product contains dUDP (< 12.5 µM in our experiment) and 32Pi. If used as a tracer (>1:100 ratio), this preparation does not compromise the chemical purity of the bulk nucleotide solution. (B) 100 µM hDUT and 50 µM γ32P-dUTP were mixed (single turnover conditions) and the reaction was stopped with 1 M HCl after various incubation times. Hydrolysis was followed by measuring the relative amount of one of the hydrolysis products, the radioactive PPi. Single exponential fits to the data (solid line) with kobs = 8.2 ± 0.4 s⁻¹. Error bars represent the S.D. of three parallel measurements. (C) The reaction of 18 µM hDUTW158 with 100 µM γ32P-dUTP (5.6-fold excess) was followed in time. Linear fit to the data yielded a kobs = 2.9 ± 0.04 s⁻¹ for the steady-state. Lag or burst was not observed at the applied time resolution. Error bars represent the S.D. of three parallel measurements.

FIGURE 5. Dissociation of PPi measured by dUTP chasing in the stopped-flow. Main Panel. Solutions of (4 µM hDUTW158 + 2 mM PPi) (black trace) or (4 µM hDUTW158 + 300 µM dUMP + 2 mM PPi) (grey trace) were mixed with 0.1 or 1 mM dUTP, respectively, in the stopped-flow. The observed fluorescence intensity change reports both the dissociation of products and the interaction with dUTP. Two exponentials fit to the data with A1 = 0.053, k1 = 648 s⁻¹ for the fast phase and A2 = 0.027, k2 = 14 s⁻¹ for the slow phase (black trace), or with A1 = 0.032, k1 = 705 s⁻¹ for the fast phase and A2 = 0.0087, k2 = 20 s⁻¹ for the slow phase (grey trace) (kobs = 726 ± 46, 15 ± 0.9 PPi, 684 ± 84, 18 ± 2.5 dUMP.PPi). Inset. PPi concentration dependence of the fast phase amplitude (A1). 4 µM hDUTW158 was mixed with different concentrations of PPi either in the absence (black circles) or in the presence (gray squares) of 300 µM dUMP. dUTP-chase was accomplished by mixing with 0.1 mM or 1 mM dUTP (black circles or gray squares, respectively). Quadratic fits to the A1 data of the recorded fluorescence time courses yielded an apparent Kd of 327 ± 117 µM for PPi (black circles) and 532 ± 128 µM for PPi binding to E.dUMP (gray squares).

FIGURE 6. Kinetic modeling of the human dUTPase enzymatic cycle. (A) Taken the same example as in Fig. 3A, a time course upon mixing 7.5 µM hDUT-158W with 5.25 µM dUTP is shown, prepared for global fitting (fluorescence normalized to the apoenzyme, dead-time considered). The solid line is a global fit to the data points using the kinetic model shown in (B) and the relative fluorescence changes in TABLE 1. (B) Kinetic model of the hDUT enzymatic cycle. Daggers and stars indicate fluorescence decrease or increase compared to the apoenzyme, respectively. The rate constants shown in the model were used as parameters of the kinetic simulation (A) and are compiled in TABLE 2. For the kPM : kPM, and kM : kM rate constant pairs, only the ratios (defined by Kd values of TABLES 1-2) and the lower bounds for the rate constant pairs are known. These lower bounds were used in the numerical simulations.
as shown. Increases in the values of these rate constants (while keeping their respective ratios constant) did not cause any detectable change in the enzyme mechanism.

FIGURE 7. **Interactions of the C-terminal arm of hDUT with adjacent monomers and dUPNPP.** The arrows in the lower left part of the model depict beta strand interactions between residues 140-144 of the C-terminal arm of monomer C (atomic coloring with blue carbons) and the N-terminus of monomer A (atomic coloring with orange carbons). Residues 149-155 of monomer C are involved in contacts with side chain and main chain atoms within the conserved motif 3 of monomer A that accommodates the deoxyribose and the uracil rings of the substrate. Arm residues 155-160 contact mostly ligand atoms (stick model, atomic coloring with yellow carbons) and each other. The last C-terminal residues (160-163) engage in extensive H-bonding to the atoms of monomer B (stick model, atomic coloring with green carbons). Structural data is taken from (13), PDB ID 2HQU.
TABLE 1 Fluorescence properties of hDUT<sup>W158</sup> apoenzyme and its ligand-bound complexes

| ligand     | K<sub>d</sub> (µM) | λ<sub>max</sub> (nm) | Relative fluorescence | K<sub>sv</sub> (M<sup>-1</sup>) | V          | anisotropy   |
|------------|-------------------|---------------------|-----------------------|-----------------|------------|-------------|
| none       | -                 | 353                 | 1                     | 6.6 ± 0.12      | 0.9 ± 0.06 | 0.077 ± 0.005 |
| dUMP       | 32 ± 2            | 347                 | 0.64 ± 0.03           | 6.1 ± 0.13      | 0.4 ± 0.07 | 0.081 ± 0.003 |
| dUDP       | 12 ± 1            | 347                 | 0.59 ± 0.03           | 5.7 ± 0.10      | 0.6 ± 0.05 | 0.085 ± 0.006 |
| dUTP       | < 1               | 339                 | 0.20 ± 0.06           | -               | -          | -           |
| dUPNPP     | 5 ± 3             | 343                 | 0.40 ± 0.04           | 5.0 ± 0.09      | 0.4 ± 0.05 | 0.091 ± 0.003 |
| dUMP,PPi   | 479 ± 20          | 351                 | 1.40 ± 0.01           | 6.0 ± 0.2       | 0.9 ± 0.10 | 0.106 ± 0.002 |
| PPi        | 146 ± 15          | 351                 | 2.53 ± 0.02           | 6.8 ± 0.19      | 1.5 ± 0.08 | 0.112 ± 0.002 |
| NATA (in the absence of protein) | N.A. | 355 | N.A. | 17.4 ± 0.12 | 1.9 ± 0.02 | 0.0044 ± 0.0008 |

For comparison, respective fluorescence characteristics of N-acetyl L-tryptophanamide (NATA), representing free tryptophan, are also given. N.A., not applicable.
TABLE 2 Kinetic parameters of the hDUT enzymatic cycle

| Parameter                                      | Value       | Source experiment                                  | Figure |
|-----------------------------------------------|-------------|--------------------------------------------------|--------|
| $k_{cat}$, s$^{-1}$                           | 6.8 ± 2.0   | Steady-state proton release assay (13)            | -      |
|                                               | 6.5 ± 0.1   | Fluorescence single turnovers                      | 3 A-B  |
|                                               | 6.7 ± 0.2   | Proton release turnovers                            | 3 C    |
| $K_M$, µM                                      | 3.6 ± 1.9   | Michaeilis-Menten global fits to proton release turnovers | 3 C    |
| $k_{cat}/K_M$, M$^{-1}$s$^{-1}$                | 1.9 x 10$^6$|                                                   |        |
| $k_B$, s$^{-1}$                                | 120         | Global fit to fluorescence traces                 | 6 A-B  |
| $k_{-B}$, M$^{-1}$s$^{-1}$                     | 100         |                                                   |        |
| $k_{ISO, obs}$, s$^{-1}$                       | 20 ± 18     | Fluorescence turnovers$^a$                         | 3 A-B  |
|                                               | 24 ± 6      | dUTP chasing$^a$                                  | 5      |
| $k_{ISO}$, s$^{-1}$                            | 21.2        | Global fit to fluorescence traces                 | 6 A-B  |
| $k_{-ISO}$, s$^{-1}$                          | 3.7         |                                                   |        |
| $k_I$, s$^{-1}$                                | 5.5 ± 2.5   | Quench-flow single turnover                       | 4 A    |
|                                               | 6.4         | Global fit to fluorescence traces                 | 6 A-B  |
| $k_{MP}$, s$^{-1}$                             | 684 ± 84    | Fluorescence PPi chasing from E.dUMP.PPi          | 5      |
| $k_{-MP}$, M$^{-1}$s$^{-1}$                    | 1.42        | $k_{MP}/K_d(E.PPi for dUMP)$$^b$                   | 5, 6 A-B|
| $k_{PM}$, s$^{-1}$                             | >1000       | Fluorescence stopped-flow$^c$                      | 6 A-B  |
| $k_{-PM}$, M$^{-1}$s$^{-1}$                    | >9.5        | $k_{PM}/K_d(E.PPi for dUMP)$$^d$                   | 6 A-B  |
| $k_M$, s$^{-1}$                                | >1000       | Fluorescence stopped-flow$^c$                      | 6 A-B  |
| $k_{-M}$, M$^{-1}$s$^{-1}$                    | >31         | $k_M/K_d(E for dUMP)$$^b$                         | 6 A-B  |
| $k_P$, s$^{-1}$                                | 740 ± 66    | Fluorescence PPi chasing from E.PPi               | 5      |
| $k_{-P}$, M$^{-1}$s$^{-1}$                    | 5           | $k_P/K_d(E for PPi)$$^b$                          | 5, 6 A-B|

$^a k_{ISO, obs} = k_{ISO} + k_{-ISO}$

$^b$ K_d values for different ligands are listed in TABLE 1.

$^c$ Reaction was practically completed in the dead-time of the stopped-flow (< 1 ms).

$^d$ K_d (E.PPi for dUMP) calculated as K_d (E for dUMP)K_d (E.dUMP for PPi)/K_d (E for PPi)
FIGURE 2

A

B

Fluorescence anisotropy

K_{SV} / M^{-1}

[acrylamide] / M

NATA  apo  dUMP  dUDP  dUMP.PPi  PPi

0.0  0.1  0.2  0.3  0.4

0.00  0.02  0.04  0.06  0.08  0.10  0.12

Fluorescence anisotropy

NATA  apo  dUMP  dUDP  dUMP.PPi  PPi

Downloaded from www.jbc.org by guest on March 25, 2020
FIGURE 4

A

Step 1: NDPK phosphorylation (in EDTA)

NDPK + γ\(^{32}\)P-ATP ⇌ 32P-NDPK + ADP

Step 2: 32P-NDPK isolation

Q-Sepharose

ADP/residual γ\(^{32}\)P-ATP immobilized on Q-Sepharose

32P-NDPK

dUDP

\(γ^{32}\)P-dUTP + NDPK

Step 3: dUDP phosphorylation (in Mg\(^{2+}\))

Step 4: Phenol extraction

\(γ^{32}\)P-dUTP

B

C

\(\frac{\text{dUTP hydrolyzed (\%)} \cdot 100}{\text{t / s}}\)

\(\frac{\text{dUTP hydrolyzed (\%)} \cdot 100}{\text{t / s}}\)
FIGURE 5
**FIGURE 6**

### A

- Graph showing relative Trp fluorescence over time (t/s) from 1E-3 to 1.

### B

**Substrate binding**

- $k_B = 121 \, \mu M^{-1}s^{-1}$
- $k_{-B} = 100 \, s^{-1}$

**Isomerization**

- $k_{ISO} = 3.7 \, s^{-1}$
- $k_{-ISO} = 21.2 \, s^{-1}$

**Chemical step**

- $k_H = 6.4 \, s^{-1}$

**Product release**

- $k_{MP} = 684 \, s^{-1}$
- $k_{MP} = 1.42 \, \mu M^{-1}s^{-1}$

Chemical reactions:

- $E + dUTP \rightleftharpoons E.dUTP_{++} \rightleftharpoons E.dUTP_{+++} \longrightarrow E.dUMP.PPi^* \rightleftharpoons E.dUMP^+$

- $E.PPi^{**} \rightleftharpoons E$

- $k_p = 740 \, s^{-1}$
- $k_{-p} = 5 \, \mu M^{-1}s^{-1}$
Kinetic mechanism of human dUTPase, an essential nucleotide pyrophosphatase enzyme
Judit Tóth, Balázs Varga, Mihály Kovács, András Málnási-Csizmadia and Beáta G. Vértesy

J. Biol. Chem. published online September 11, 2007 originally published online September 11, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M706230200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts