Kinetic Characterization of dUTPase from *Escherichia coli*\*  

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The enzyme dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate, thereby preventing a deleterious incorporation of uracil into DNA. The best known dUTPase is that from *Escherichia coli*, which, like the human enzyme, consists of three identical subunits. In the present work, the catalytic properties of the *E. coli* dUTPase were investigated in the pH range 5–11. The enzyme was found to be highly specific for dUTP and discriminated both base and sugar as well as the phosphate moiety (bound dUDP was not hydrolyzed). The second best substrate among the nucleotides serving as building blocks for DNA was dCTP, which hydrolyzed an astonishing 10⁵ times less efficiently than dUTP, a decline largely accounted for by a higher *Kₘ* for dCTP. With dUTP-Mg as substrate, *kₐₚ* was found to vary little with pH and to range from 6 to 9 s⁻¹. *Kₘ* passed through a broad minimum in the neutral pH range with values approaching 10⁻⁷ M. It increased with deprotonation of the uracil moiety of dUTP and showed dependence on two ionizations in the enzyme, exhibiting *pKₐ* values of 5.8 and 10.3. When excess dUTPase was reacted with dUTP-Mg at pH 8, the two protons transferred to the reaction medium were released in a concerted mode after the rate-limiting step. The Mg²⁺ ion enhances binding to dUTPase of dUTP by a factor of 100 and dUDP by a factor of 10. Only one enantiomer of the substrate analog 2'-deoxyuridine-5'-(α-thio)-triphosphate was hydrolyzed by the enzyme. These results are interpreted to favor a catalytic mechanism involving magnesium binding to the α-phosphate, rate-limiting hydrolysis by a shielded and activated water molecule, and a fast and ordered desorption of the products. The results are discussed with reference to recent data on the structure of the *E. coli* dUTPase-dUDP complex.

The enzyme dUTPase (EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate and is an important factor for the prevention of uracil incorporation into DNA (1). Along with the reduced nucleotides required for the synthesis and repair of DNA, the ribonucleotide reductase converts UDP to dUDP. The latter becomes phosphorylated to dUTP, which is readily mistaken for dTTP by DNA polymerase and, hence, may become incorporated into DNA (2). The adverse effects of a low dUTPase activity suggest that inhibitors of this enzyme may find a role as cytostatic drugs in cancer therapy (4). dUTPase is widespread in nature and has been found in a variety of eukaryotic and prokaryotic organisms as well as in many viruses (5–8). It has been proven to be essential for the viability of *Escherichia coli* (9) and *Saccharomyces cerevisiae* (10). In some organisms, a second major role for dUTPase is to provide dUMP for the synthesis of dTTP (11).

The three-dimensional structure of dUTPase from *E. coli* has been determined to 1.9-Å resolution (12) and reveals a trimeric architecture of identical subunits, in conflict with an earlier report of dUTPase being a tetramer (2). A recent study on the structure of the *E. coli* dUTPase-dUDP complex indicates that the substrate-binding pockets are at the interface of two subunits (13). The amino acid sequence of dUTPases from various species show a high degree of similarity (14). Most of the conserved residues are located in five regions of the polypeptide chain, which line the active site in the *E. coli* enzyme (13). This finding suggests that dUTPases from various organisms operate by the same catalytic mechanism. The *E. coli* dUTPase was first reported to be a zinc metalloenzyme (2), but Hoffmann (15) concluded that the Mg²⁺ ion serves as a cofactor for the enzyme, consistent with dUTPases from other species being reported as Mg²⁺-dependent enzymes (5).

In spite of the importance of dUTPase for the reproduction of the cell, the potential role of the enzyme as a target for drugs in cancer therapy, and the recent successes in determining its structure, the functional properties of dUTPase are poorly characterized. A main obstacle to progress in this field was removed by the development of techniques to amplify the expression of dUTPase in *E. coli* (16). It remained, however, to develop sensitive methods for monitoring the optically transparent dUTPase reaction. In the past, the activity of dUTPase has been measured by separation and quantitation of radioabeled reaction products by TLC (3) or by column chromatography (15). These methods are discontinuous and slow, which may explain the large variation in the values reported for the kinetic parameters of the dUTPase from *E. coli* (2, 11, 16).

Rogers et al. (1) took advantage of the fact that hydrolysis of dUTP to dUMP and PPi is coupled to a pH-and PMg-dependent release of protons and used the pH-stat to measure the dUTPase reaction. Their measurements at pH 7 gave a *kₐₚ* of 6 s⁻¹ and a *Kₘ* in the range of 0.3–0.5 µM, in close agreement with the corresponding values presented in this work. Using water labeled with ¹⁸O, they could further show that dUMP, and not PPi, is transferred to water in the dUTPase reaction.

In the present work, we extended the use of the stopped-flow pH indicator technique (17) to monitor complete progress curves for the dUTPase reaction and to measure its kinetic parameters in the pH range 4–11. The results provide evidence...
that dUTPase from *E. coli* has evolved close to maximal catalytic efficiency toward dUTP and effective means to exclude the other nucleotides. This enzyme therefore appears extremely fit to prevent incorporation of uracil into DNA.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation**—dUTPase was isolated from *E. coli* strain pHW1, pRK248/MC1000 according to a method described by Hofmann et al. (16) and stored at −20 °C in 25 mM Bis-Tris, 100 mM NaCl, pH 7.0. Prior to each measurement, the enzyme solution was desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated with 1 mM acetic acid, pH 5.8, in 0.1 M KCl. The protein concentration was determined spectrophotometrically at 280 nm using the extinction coefficient of 0.51 ml mg⁻¹ (15). The dUTPase active-site concentration was determined kinetically from independent estimates of *k*₉,cat and *V*₉,cat as described under “Results.”

**Chemicals**—The nucleotides dCTP, dTP, dUMP, and dUDP were purchased from Sigma. dTTPs, dUTP, and UTP were from Pharmacia; uracil and uridine were from Fluka Chemie AG (Buchs, Switzerland). dCTPase² was deaminated to dUTPase by a method described by Lohmann (18). The nucleotides were purified on 4 ml of TSK-DEAE-650(S) (Merck, Darmstadt, Germany), equilibrated with 0.01 M HCl. Pure fractions were eluted with a 60-ml gradient of 0.01–0.2 M HCl in 0.01 M KCl, subsequently adjusted to pH 6.5 with KOH and stored at −70 °C. The pH indicators bromthymol blue, bromcresol purple, chlorphenol red, cresol red, and alizarine yellow were obtained from Merck. Stock solutions containing about 1 mm indicator were titrated spectrophotometrically with NaOH at a wavelength that gave a final absorbance value of less than 2. Other chemicals used in this investigation were of the highest available quality.

**Kinetic Measurements**—Most kinetic measurements were performed with a Durrum D-100 stop-flow spectrophotometer, modified to prevent syringe leakage and connected to a computer for data acquisition and storage. Unless otherwise stated, the measurements were performed at 25 °C in degassed solutions containing 5 mM MgCl₂ and adjusted to 0.1 M ionic strength with KCl. Protons, liberated through hydrolysis of dUTP, were neutralized by a pH indicator and a buffer with similar values of extinction coefficient, absorbance, and extinction coefficient at the absorbance peak of the basic form of the indicator. The indicator:buffer ratio was typically 25:250 (μM) but was modified in response to the number of protons expected to be released in the reaction, the extinction coefficient of the indicator, and the buffer capacity of the solution. In most experiments, the pH changes were kept within 0.05 units to ensure signal linearity (see Appendix). The specific pairs of pH indicator and buffer used were chlorophenol red or bromcresol purple in combination with MES (pH 5.0–6.4), bromthymol blue or MOPS (pH 6.0–7.2), cresol red with Bicine (pH 7.4–8.6), and alizarine yellow with CHES or CAPS (pH 9.0–10.3). The effects of these indicators and buffers on the dUTPase reaction were tested by varying the indicator concentrations and extinction coefficient of each buffer pair at the same pH. Prior to each measurement, the apparent *p*Kᵦ and extinction coefficient of the indicator were determined by direct titration in the stopped-flow spectrophotometer. For this purpose, the indicator was dissolved in a solution that, except for enzyme and substrate, had the same composition as the assay solution. In this way, the pH of the latter could be measured from the absorbance subsequently measured in the assay. Solutions containing pH indicator and enzyme were used to continuously monitor the dUTPase reaction at pH values above 5. Enzyme, typically at a concentration of 0.1 μM active sites, was reacted with 1–5 μM substrate in the stopped-flow reaction chamber. Indicator absorbance changes corresponding to complete hydrolysis of dUTP were recorded, and the kinetic parameters were evaluated by application of the integrated Michaelis-Menten equation, as detailed under “Results.”

Below pH 5, hydrolysis of dUTP occurs without a significant release of protons, and the indicator method cannot be applied directly. Instead, slow hydrolysis of dUTP was initiated in one syringe of the stopped-flow apparatus by mixing substrate (2–10 μM) with small amounts of enzyme (2–5 nM) and 1–10 mM acetic acid serving as buffer. The stopped flow was then activated at timed intervals to react residual dUTP with additional enzyme (150 nM) in the presence of 25 μM cresol red, 0.25–2 mem Bicine, and KOH sufficient to jump pH to about 8. The course of the slow reaction was determined by plotting the amplitudes of the pH-fluorescence changes against time. The kinetic parameters were evaluated by the integrated Michaelis-Menten equation. Single-turnover measurements were performed by reacting excess dUTPase (5 μM) with dUTP (1 μM) in the presence of cresol red and Bicine.

To ascertain that the dUTPase reaction goes to completion under the conditions tested in this investigation, dUTP, MgCl₂, and dUTPase were studied and analyzed for residual dUTP by ion exchange chromatography.

**Titration of dUTP**—The acid dissociation constants of the triphosphates (p*Kᵦ*) and uracil moieties of dUTP were determined by potentiometric titration of 0.3 mM dUTP in 0.1 M KCl, using KOH as titrator.

**Metal Ion Requirement**—The dependence of the activity of *E. coli* dUTPase towards metal ions was investigated by including various amounts of EDTA, MgCl₂, or CaCl₂ in reaction mixtures that contained endogenous metal ions.

**Inhibition**—Inhibition of dUTPase by the products dUMP and PPᵢ, individually or combined at equimolar concentrations, as well as by dUDP, UTP, dCTP, and dTTP was studied at pH values close to 8 (dUMP in addition at pH 5). For this purpose, various concentrations of the potential inhibitor were included in the assay solutions, described in the previous section. The inhibitor constants were evaluated by linear least-square analysis of plots of *K* against inhibitor concentration. Inhibition of dUTPase by the metal-free form of dUTP was examined at pH 7.8 using the cresol red/Bicine buffer system. The enzyme, 0.5 μM active sites, was reacted with 10 μM dUTP in the presence of 0.5 and 0.75 mM MgCl₂, respectively. The reactions were repeated after the addition of 1 mM EDTA to reduce and buffer the concentration of free Mg²⁺. As outlined under “Results,” the effect of EDTA on the kinetic parameters can be used to calculate the inhibition constant for metal-free dUTP. Inhibition by the metal-free form of dUDP was similarly studied in the presence of 0.5 μM MgCl₂ and 1 mM EDTA. The concentrations of unbound EDTA in these experiments were sufficient to reduce the activity of dUTPase caused by endogenous metal ions to an insignificant level.

**Hydrolysis of Other Nucleotides by dUTPase**—The hydrolytic activity of dUTPase toward the magnesium complexes of dUDP, UTP, dCTP, dTTP, and dUDP was studied by incubating the nucleotides (0.6–1.0 mM) at pH 7–8 with and without dUTPase (2–6 μM). After 1–2 h of incubation, the mixtures were analyzed by ion exchange chromatography using a fast protein liquid chromatography system equipped with a Mono Q HR column (Pharmacia). The chromatography was developed with 0–0.6 M KCl in 10 mM MOPS at pH 7. For dCTP, the time course of the reaction was monitored by taking samples from the incubation mixture at regular intervals. The dUTPase activity in the samples was quenched with 10 mM EDTA, and the nucleotides were quantitated by chromatography on Mono Q followed by peak integration. A first-order decay function was fitted to the data for residual dCTP, and the specificity constant (k₉/Kᵦ) was determined by dividing the observed rate constant by the concentration of enzyme. The kinetic parameters for hydrolysis of the diastereomeric substrate analog dUTPαS by dUTPase were determined for a series of concentrations of the analog by the pH indicator method.

**RESULTS**

**Basic Kinetic Characterization**—When pH indicators are used to monitor release or consumption of protons in buffered solutions, the change in indicator absorbance is essentially linear with product formation if the *p*Kᵦ values of the indicator and the buffer are close to identical and the combined concentration of indicator and buffer is much higher than the sum of [H⁺] and [OH⁻]. If these requirements cannot be satisfied, as was typically the case in this investigation, it becomes important to keep the pH changes small and to use the appropriate expression to estimate the deviation of the signal from linearity. This aspect of the investigation is outlined in detail in the Appendix. For here, it suffices to mention that the data used for evaluation of the kinetic parameters were sampled in an interval of the reaction traces over which deviation from a linear response in absorbance to formation of product was less than 3%.

Control measurements, described under “Experimental Procedures,” showed that the pH indicators and buffers employed in this investigation do not have a significant effect on the
When initiated with enzyme in a large excess over substrate, the reaction in Scheme 1 will complete a single turnover by two exponential transients. These correspond largely to accumulation and breakdown of the enzyme substrate complex, respectively, and are assigned time constants \( r_1 \) and \( r_2 \). The latter are the roots of a quadratic equation, which, if the roots are real and differ considerably in magnitude, has the following approximate solutions.

\[
\begin{align*}
r_1 & = k_{\text{cat}} C_E \left( 1 + \frac{K_m}{C_E} \right) \quad (\text{Eq. 3}) \\
r_2 & = \frac{k_{\text{cat}}}{1 + \frac{K_m}{C_E}} \quad (\text{Eq. 4})
\end{align*}
\]

In these expressions, \( K_m \) is \((k_{-1} + k_{\text{cat}} k_{\text{diss}})\) (as under steady-state conditions) and \( C_E \) is the total concentration of enzyme. With \( C_E \gg K_m \), accumulation of ES will be close to stoichiometric, and \( r_1 \) and \( r_2 \) reduce to \( k_{\text{cat}} C_E \) and \( k_{\text{cat}} \), respectively. An absorbance trace obtained by reacting 5 \( \mu \)M of dUTPase active sites (25 times \( K_m \)) with 1 \( \mu \)M dUTP-Mg in the presence of cresol red is shown in Fig. 2. As demonstrated in the Appendix, the absorbance change monitored corresponds to the release of two protons for each dUTP hydrolyzed. From the definition of \( V_{\text{max}} \) and \( K_m \), it follows that \( k_{\text{cat}} C_E \gg V_{\text{max}}/K_m \). Hence, based on the values for the kinetic parameters reported above and the concentration of enzyme used, the faster of the two transients (\( r_1 \)) should have a half-life of less than 6 ms. The single-exponential trace observed has a half-life of 125 ms and must represent the second, slower relaxation (\( r_2 \)). At the concentration of enzyme tested \( (C_E \gg K_m) \), this corresponds to breakdown of the enzyme-substrate complex into free enzyme and products at a rate approaching \( k_{\text{cat}} \). Evaluation of the data in Fig. 2 gave \( k_{\text{diss}} = 5.5 \text{ s}^{-1} \), which, according to Equation 4, gives \( k_{\text{cat}} = 5.8 \text{ s}^{-1} \). The enzyme active-site concentration was determined by repeating the reaction, but with substrate in excess over enzyme. From the observed value of \( V_{\text{max}} \), the concentration of enzyme active sites was calculated using the relationship \( C_E = V_{\text{max}}/k_{\text{cat}} \).

**Effects of pH on \( k_{\text{cat}} \) and \( K_m \)**—The steady-state kinetic parameters, \( k_{\text{cat}} \) and \( K_m \), were determined in the pH interval 5–10.3 using the methods described under “Experimental Procedures.” With the exception of a minor decrease in the pH interval 6–7, \( k_{\text{cat}} \) was found to be relatively insensitive to pH and to vary from 9 s\(^{-1} \) (pH < 6) to 5.8 s\(^{-1} \) (pH > 7). The corresponding data for \( K_m \) are presented in Fig. 3 and show that substrate binding is affected by at least three proton dissociation equilibria according to the following relationship.
Inhibition of dUTPase—In the presence of a substrate-competitive inhibitor, K in Equation 2 equals $K_m(1 + C/K_c)$, where $K_m$ refers to dUTP-Mg, $C$ is the total concentration of the inhibitor, and $K_c$ is the dissociation constant for the enzyme-inhibitor complex. A plot of $K_m$ against $C$ should yield a straight line that intersects the $y$ axis at $K_m$ and the $x$ axis at $-K_c$. Inhibition of dUTPase by dUDP in the presence of 5 mM Mg$^{2+}$ is demonstrated in Fig. 4. The traces shown were obtained with 2 $\mu$M dUTP in the absence and presence of 20 $\mu$M dUDP. The linear dependence of the apparent $K_m$ on the concentration of dUDP, demonstrated by the inset, is consistent with competitive inhibition of dUTPase by dUDP with a $K_c$ of 15 mM. Data for the competitive inhibition of dUTPase by other nucleotides and PP$\_1$Mg are summarized in Table I.

Metal Ion Requirements—Without added MgCl$_2$, an apparent $K_m$ of 2 $\mu$M was observed for hydrolysis of 2 $\mu$M dUTP by the E. coli dUTPase at pH 8. This value increased to about 15 $\mu$M after the addition of 20 $\mu$M EDTA, indicating that endogenously present metal ions were responsible for the observed activity. The addition of 0.5 mM CaCl$_2$, instead of EDTA, had a slightly increasing effect on the $K_m$ value, showing that Ca$^{2+}$ is not a cofactor for the E. coli dUTPase and does not, at the concentration tested, compete successfully with endogenous metal ions for binding to dUTP. No change in the apparent $K_m$ could be observed when the concentration of the Mg$^{2+}$ was raised from 0.5 to 5 mM, indicating that a dUTPase-Mg complex, with the metal in the substrate pocket, must have a dissociation constant higher than 5 mM.

Metal Ion Buffering and Inhibition by Metal-free dUTP—With 0.5 mM MgCl$_2$ and 1.0 mM EDTA in the assay solution, the concentration of free magnesium ion is buffered at about 0.66 $\mu$M, the value reported for the dissociation constant of the EDTA-Mg complex at pH 8 (20). The addition of 20 $\mu$M dUTP to the system will change this concentration by less than 0.001 $\mu$M. Consequently, the hydrolysis of dUTP to dUMP and PP$_1$ will have insignificant effects on the concentration of free Mg$^{2+}$, in particular since PP$_1$ binds Mg$^{2+}$ essentially as strongly as does dUTP. When MgCl$_2$ is raised from 0.5 to 0.75 mM, the buffered concentration of free Mg$^{2+}$ increases by a factor of 3. At the low and buffered concentration of free Mg$^{2+}$ resulting from the addition of excess EDTA to the assay system, the concentration of metal-free dUTP is approximately equal to the total concentration of substrate. As a consequence, the slope of the progress curve for the dUTPase reaction is given closely by the equation,

$$\frac{d[dUTP]}{dt} = \frac{V_{\text{max}}}{1 + \frac{K'}{[\text{dUTP}]}(1 + \frac{[\text{dUTP}]}{K'})}$$

(Eq. 6)

where $K_c$ is the inhibition constant for metal-free dUTP and $K'$ is defined as follows.

$$K' = K_{\text{dUTP-Mg}}$$

(Eq. 7)

In the latter expression, $K_m$ is that for dUTP-Mg, and $K_{\text{dUTP-Mg}}$ is the dissociation constant for this complex. Therefore, when Equation 2 is applied for evaluation of the progress curves obtained in the presence of EDTA, the kinetic parameters evaluated will correspond to the following.

$$V = \frac{V_{\text{max}}}{1 + \frac{[\text{dUTP}]}{K'}}$$

(Eq. 8)
suggested that the displacing water molecule becomes trapped in the substrate pocket on formation of the dUTPase–dUTP complex and is shielded from the surrounding medium. Because the water molecule must expel one of its protons to displace pyrophosphate from the α-phosphorus of dUTP, such an arrangement requires the presence of a base in the active site, which can temporarily harbor the proton and activate the attacking water. Recent data on the structure of the dUTPase–dUDP complex (13) suggests that an aspartate residue (Asp-90), evolutionarily conserved among dUTPases, may play such an activating role. The charge of Asp-90 would be partly neutralized by a hydrogen bond to the substrate water molecule, and its pKₐ in the enzyme-substrate complex should therefore be well below 5, consistent with the absence of a major effect of pH on kcat above pH 5. In the free enzyme, the pKₐ of Asp-90 is expected to be high for an aspartate residue because of its close proximity to Asp-92 (13). Hence, protonation of Asp-90 in the free enzyme could account for the pKₐ = 5.8 pH effect on Kₐ, since productive binding of dUTP and water would require deprotonation of the carboxylate group. In the alkaline pH range, Kₐ was affected by two ionizing groups. The more acidic of the two was shown to ionize in parallel with, and was therefore identified as, the uracil moiety of dUTP. A possible candidate for the second group, with an apparent pKₐ of 10.3, is the conserved Tyr-93 that lines the deoxyribose subsite in the dUTPase–dUDP complex (13).

Proton Release and the Rate-limiting Step—Because there was no effect of pH on Kₐ in the neutral range, formation of the productive dUTPase–dUTP–Mg complex occurs without release or uptake of protons. The turnover number did not decrease (it increased slightly) when the hydrogen ion concentration was raised by a factor 10³. This suggests that formation of the enzyme-bound products also occurs without exchange of protons. Nevertheless, at pH 8 and saturating concentrations of Mg²⁺, both protons carried by the substrate water are ultimately released to the reaction medium (22). The single-turnover experiment was designed to monitor these protons. No lag or burst could be observed, consistent with a concerted fast release of both protons after the rate-limiting step. Our interpretation is that the dUTPase reaction is limited by the step(s) that leads from enzyme-bound substrate to enzyme-bound products and that the protons are released at a subsequent fast desorption of the reaction products from the enzyme surface. The assumption about the rate-limiting step is supported by the 100-fold reduction of kcat when dUTP was used as substrate. Also, desorption of dUMP from the enzyme is not likely a rate-limiting factor. A rate of 10⁻¹ s⁻¹ or less for this step would imply a second order rate constant for association of dUMP on the order of 10⁻⁴⁻¹⁰⁻⁵ s⁻¹ (based on a Kₐ for dUMP of 2.4 mM), which is about 10⁴ times lower than that for productive association of the more complex substrate molecule.

Product Inhibition and Metal Binding—If dissociation of dUMP and PP_i from the active site is ordered, the product that leaves the enzyme last will act like a substrate competitive inhibitor with effects exclusively on Kₐ, whereas accumulation of the first leaving product will affect both Vmax and Kₐ. When tested individually, in the presence of 5 mM MgCl₂, both dUMP and PP_i acted like weak substrate competitive inhibitors, with no discernible effect on Vmax. This could be the result of a rapid random equilibrium of the product dissociation steps. An alternative explanation is that the binding sites for dUMP and PP_i overlap. This would occur if PP_i-Mg, with the metal chelated between the two phosphates, binds to the α,β-subsite, rather than the β,γ-subsite, in the substrate pocket. With such overlapping binding, the simultaneous presence of dUMP and PP_i-Mg should have the same effect on Kₐ as a single substrate-
competitive inhibitor. The associated overall inhibition constant should be related to the inhibition constants for dUMP (K_i = 1.5 mM) and PP_i-Mg (K_i = 2.5 mM) (Table I) through K_{i(app)} = K_iK_2/K_m + K_2, and, hence, amount to about 0.9 mM. Indeed, the apparent K_i exhibited a linear dependence on equimolar concentrations of dUMP and PP_i-Mg, and the inhibition constant observed was 0.7 mM (data not shown). With rapid random equilibration of the product-releasing steps, a nonlinear response in K_i is expected when the concentrations of the two products are co-varied. In view of these results, and the clear cut competitive inhibition observed for dUMP, our interpretation is that the reaction is preferentially ordered, with dUMP being the last leaving product.

Regarding the nature of the enzyme-bound products, these could be dUMPH and PP_i-Mg with the metal ion bound in between. In this position, Mg^{2+} would reduce the electrostatic repulsion between the two substrates for the reverse reaction. Formation of dUTP would depend on the protonated form of the postulated carboxylate (Asp-90), now acting as an acid catalyst in promoting extraction of neutral water. In binding of dUTP, the metal ion bound in the α, β-position could reduce repulsion by the negatively charged Asp-90 or Asp-92. The 100-fold stronger binding of dUTP-Mg to the active site compared with metal-free dUTP, the 10-fold stronger binding of dUMPH compared with dUMP^2− and of dUDP-Mg compared with dUDP^2− (Table I) are observations consistent with this idea. This role for the metal ion implies interactions with both substrate and enzyme in the Michaelis complex. Accordingly, our data show that Ca^{2+} does not promote hydrolysis of dUTP by dUTPase. The Ca^{2+} ion may or may not replace Mg^{2+} as cofactor in enzymic reactions involving nucleotides (23). The failure of Ca^{2+} to do so has been taken to indicate binding of the metal ion as a bridge between the enzyme and the nucleotide substrate where the larger Ca^{2+} would distort the binding of the substrate (23, 24).

Enzyme Efficiency—The value obtained for k_{cat}/K_m in the E. coli dUTPase system, 3 × 10^{7} M^{−1} s^{−1}, is remarkably high but is still 10 times lower than the highest k_{cat}/K_m values reported (25), considered to approach the limit set by diffusion. In aqueous solution, Mg^{2+} coordinates all three phosphates of ATP (24, 26). By inference, aqueous dUTP-Mg should exist as four different enantiomers with respect to the configuration at the α and β-phosphorus atoms. This can reduce k_{cat}/K_m in the dUTPase system by a factor of 4, since only one of these enantiomers could have a configuration complementary to the active site of dUTPase. Rearrangements required to convert the encounter complex to the productive Michaelis complex, or conformational equilibria in the free enzyme, account for any further reduction of k_{cat}/K_m relative to diffusion-controlled formation of the Michaelis complex.

Substrate Properties of dUTPase—One enantiomer of the diastereoisomeric dUTPases was found to be a substrate for dUTPase with relatively tight binding (K_m ≈ K_f = 0.9 μM), whereas the other was not hydrolyzed and did not appear to bind to the substrate pocket with a dissociation constant below 50 μM. This is most easily understood in view of a model where Mg^{2+} binding to the α-phosphate is required in the productive enzyme-substrate complex. Metal binding to the chiral α-phosphate in dUTPases imposes drastic differences between its R- and S-forms with regard to the constraints of the active site. Because Mg^{2+} does not bind easily to sulfur (27), only one of the enantiomers of the dUTPase should be able to bind the metal in the correct position for enzyme binding and catalysis. The two enantiomers generated by Mg^{2+} binding to the prochiral α-phosphate in native dUTP interchange rapidly, and all dUTP is hydrolyzed at a single rate.

The most conspicuous catalytic properties of dUTPase are its almost exclusive specificity for dUTP and slow turnover. The next best substrate among the nucleotides that serve as building blocks for DNA was dCTP, which was hydrolyzed 2 × 10^{4} times less efficiently than dUTP. The inhibition constant measured for dCTP was 4 mM and should equal the K_m for hydrolysis of this nucleotide by dUTPase. With K_m = 5.7 kcal mol^{−1} contributed by the uracil relative to the cytosine moiety is used almost exclusively for tight binding of dUTP and not for a reduction of the activation energy of the chemical step. The utilization of binding energy for a parallel increase in k_{cat} and K_m effectively increases the reaction rate only as long as K_m is lower than the resulting substrate concentration. Hence, a simple explanation to the low k_{cat} and K_m values found for dUTPase would be that K_m is already higher than the concentration of dUTP in the E. coli cell.

The dual role of dUTPase is to remove dUTP and to be virtually unreactive with the other nucleotides. The ability of the enzyme to discriminate dCTP and dUTP appears most critical, since the two pyrimidines are similar in shape. The different hydrogen bonding properties of the two bases, which govern their incorporation into RNA/DNA, may also determine the specificity of dUTPase for the uracil moiety. Our observation that ionization of the pyrimidine ring in dUTP prevents binding to the active site is consistent with this idea; exclusion of the ionized form would result from disruption of the hydrogen bonding pattern. We assume that the uracil residue is too narrow to accommodate the methyl group of thymine and that the enzyme exploits the difference between uracil and cytosine by forming donor and acceptor hydrogen bonds, respectively, to the C-4 carbonyl oxygen and the N-3 hydrogen of the former. Strong interactions with the C-2 carbonyl group are not expected, since this would not increase the specificity for uracil over thymine and cytosine and could result in such tight binding that release of dUMP becomes rate-limiting for the reaction. This view is consistent with data on the structure of the dUTPase-dUDP complex (13). The need for an enzyme that can discriminate not only the uracil moiety from other closely related bases, but also the deoxyribose from ribose is easily understood, since this will prevent a wasteful and possibly fatal hydrolysis of nucleotides needed for the synthesis of DNA and RNA.

Measurements at pH 4 confirmed that dUTPase from E. coli is active at this pH. This is an important observation, since the crystals used for structural determination of dUTPase were grown in the pH 4–5 range (13, 28).

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APPENDIX

Protons released into an aqueous solution of pH indicator and buffer partition between the conjugate bases of the indicator, buffer, and water. The change in indicator absorbance (A), caused by a proton-releasing (or consuming) reaction in such a system, is described by the equation,

\[ \frac{dA}{d[P]} = n\Delta e\epsilon \frac{1}{\lambda} \]  
(Eq. 10)

where P is product, n is the number of protons released (n < 0) or taken up (n > 0) for each product molecule formed, \( \Delta e \) is the difference between the molar extinction coefficients of the basic and acidic forms of the indicator at the wavelength studied, and
$\ell$ is the length of the optical path. The factor $q_i$ is the fraction of the released protons that binds to the indicator. It is equivalent to the relative buffer capacity of the indicator as expressed by Equation 11.

$$q_i = \frac{C_{\text{ind}} (1 + [H^+]^{-2})}{K_{\text{ind}} (1 + [H^+]^{-2}) + C_{\text{buf}} (1 + [H^+]^{-2}) + K_{\text{buf}} [H^+]^{-1}}$$  \hspace{1cm} (Eq. 11)

In this expression, $K_{\text{ind}}$ is the ionization constant of water, and $K_{\text{buf}}$ denotes total concentration and $K$ denotes acid dissociation constants, as indicated by subscripts. Buffer capacity, conferred by additional components in the system, can be accounted for by adding the corresponding expression to the denominator. The expression in Equation 11 is approximately equal to $C_{\text{ind}}/(C_{\text{ind}} + C_{\text{buf}})$ when $K_{\text{ind}} = K_{\text{buf}}$ and $C_{\text{ind}} + C_{\text{buf}} \gg [H^+] + [OH^-]$. If these conditions are not fulfilled, $q_i$ may still be treated as a constant over small changes in pH. For instance, using data presented below, it is estimated that $q_i$ changes by no more than 3% in the course of the reaction demonstrated in Fig. 1, in spite of the 0.4 units difference between the pH values of the buffer and indicator used. Hence, over small changes in pH, the absorbance change can be quantitatively evaluated using Equation 12.

$$\Delta[P] = \frac{\Delta A}{n \Delta \ell q_i}$$  \hspace{1cm} (Eq. 12)

Direct spectrophotometric titration of the assay solution for dUTPase, containing cresol red and Bicine, gave pH

\[ \text{pH [Durrum]} = 7.9 \]

and $\Delta A = 34,000 \text{ m}^{-1} \text{ cm}^{-1}$ at 573 nm. Based on these values and an optical path in the Durrum instrument of 2 cm, the pH values at the midpoints of the three reactions shown in Figs. 1, 2, and 4 are calculated to be 7.56, 7.72, and 8.56, respectively, and the corresponding total changes in pH are calculated to be 0.044, 0.053, and 0.076 units. Since dUTP was completely hydrolyzed and $q_i$ was approximately constant over these pH changes, Equation 12 can be used to calculate the number of protons released in the respective reaction. With $pK_{\text{buf}} = 8.26$ for Bicine, the absorbance changes reported in Figs. 1, 2, and 4 correspond to the release of 2.0, 1.8, and 2.1 protons, respectively, for each dUTP hydrolyzed. These figures and those in Table II, which compile data for proton release in the dUTPase reaction measured at various pH and pMg values as well as with different indicator/buffer pairs, are consistent with the data presented by Alberty (22) for the analogous hydrolysis of ATP and with the data obtained by Rogers et al. (1) for hydrolysis of ATP.

**Table II**

<table>
<thead>
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<th>pH</th>
<th>pMg</th>
<th>n</th>
<th>Indicator/Buffer</th>
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<tbody>
<tr>
<td>5.9</td>
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<td>0.36</td>
<td>BCP/MES</td>
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</tr>
<tr>
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<td>0.76</td>
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<td>BTB/MOPS</td>
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<tr>
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<td>5.3</td>
<td>1.28</td>
<td>CR/Bicine</td>
</tr>
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<tr>
<td>7.5</td>
<td>3.3</td>
<td>1.94</td>
<td></td>
</tr>
</tbody>
</table>

*Indicators were bromocresol purple (BCR), bromothymol blue (BTB), and cresol red (CR).*

**REFERENCES**

Kinetic Characterization of dUTPase from *Escherichia coli*
Gunilla Larsson, Per Olof Nyman and Jan-Olov Kvassman

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