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Deoxythymidine Triphosphate–Deoxyuridine Triphosphate Nucleotidohydrolase Induced by *Bacillus subtilis* Bacteriophage φe†

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ABSTRACT: Deoxythymidine triphosphate nucleotidohydrolase (dTTPase)–deoxyuridine triphosphate nucleotidohydrolase (dUTPase), a membrane-associated enzyme induced after bacteriophage φe infection of *Bacillus subtilis*, has been solubilized, partially purified, and characterized. Similarities in kinetics of induction, pH optimum, magnesium and thiol requirement, sensitivity to *p*-hydroxymercuribenzoate, molecular weight, and purification suggest that both dTTPase and dUTPase activities reside in a single protein. Kinetic analysis revealed that dTTP and dUTP are hydrolyzed at the same active site and have K_M's of 400 and 11 μM, respectively. The substrate specificity is consistent with the hypothesis that the enzyme plays a role in the depletion of the intracellular pools of dTTP and/or dUTP after φe infection and thus prevents the incorporation of thymine and/or uracil into the hydroxymethyluracil-containing DNA of phage φe.

φe is one of a group of *Bacillus subtilis* bacteriophages unique because their DNA contains the unusual base 5-hydroxymethyluracil (HMU)[1] rather than thymine (Roscoe and Tucker, 1966). The HMU-DNA *B. subtilis* phages are similar in morphology, size, and DNA content (Green, 1964; Truffaut *et al.*, 1970) to the *Escherichia coli* T-even phages whose DNA contains HMC rather than cytosine (Wyatt, 1955). Like the T-even phages, phage φe induces several proteins (see Figure 1) after infection which alter the host's deoxyribonucleotide metabolism, in this case to allow the synthesis of HMU-DNA and to prevent the synthesis of DNA containing thymine or uracil.

Kahan *et al.* (1964) and Roscoe (1969a) have reported the induction after HMU-phage infection of nucleotidohydrolase activities which catalyze the conversion of deoxythymidine triphosphate (dTTP) and deoxyuridine triphosphate (dUTP) to dTMP and dUMP plus pyrophosphate. The dTTP nucleotidohydrolase (dTTPase) activity was thought to be responsible for excluding thymine from phage DNA. In addition, dUTPase was postulated to function in supplying dUMP as substrate for dUMP hydroxymethylase. Roscoe (1969a,b) presented preliminary genetic and biochemical evidence that the φe-induced dTTPase and dUTPase activities might reside in a single enzyme. This report describes the partial purification and characterization of the φe-induced dTTPase–dUTPase and confirms Roscoe's hypothesis that a single enzyme possesses both activities. The properties of the partially purified enzyme were examined for consistence with the presumed role of the enzyme *in vivo* and to allow a direct comparison with the properties of the deoxycytidine triphosphate nucleotidohydrolase (dCTPase–dUTPase) induced after T-even phage infection of *E. coli* (Price and Warner, 1969).

Materials and Methods

Microorganisms. Bacillus subtilis N.C.T.C. 3610 and phage φe were kindly provided by S. E. Luria. Conditions for the growth of cells and phage were those of Roscoe and Tucker (1966).

Biochemicals. [5-^{3}H]dUTP was purchased from Mallinckrodt and repurified on Dowex AG-1-X8 resin by the method of Brown (1972). [*Methyl*-^{3}H]dTTP was from Schwarz/Mann, and thymidine 5′-tetraphosphate and β,γ-methylene-dTTP were from Sigma. The 4-thio-dTTP was the gift of Dr. Karl Scheit, and *Micrococcus lysodeikticus* DNA was the gift of Dr. Robert Armstrong.

Cell Extract Preparation. B. subtilis cells were grown by vigorous shaking at 37° to a density of 1×10^8 colony-

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[1] Abbreviations used are: HMU, 5-hydroxymethyluracil; HMC, 5-hydroxymethylcytosine; dTTPase–dUTPase, deoxythymidine triphosphate–deoxyuridine triphosphate nucleotidohydrolase; HSEtOH, 2-mercaptoethanol; HgBzOH, *p*-hydroxymercuribenzoate.

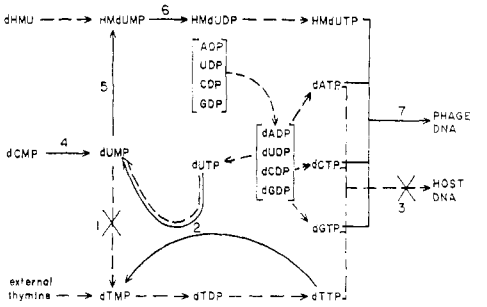

FIGURE 1: Proposed scheme for deoxyribonucleotide metabolism after HMU phage infection of *B. subtilis* (host enzyme, dashed arrows; phage-induced enzyme, solid arrows), indicating phage-induced proteins: (1) thymidylate synthetase inhibitor (Haslam *et al.*, 1967); (2) dTTPase-dUTPase (Kahan *et al.*, 1964; Roscoe, 1969a); (3) bacterial DNA arrest protein (Marcus and Lavi, 1972); (4) dCMP deaminase (Roscoe and Tucker, 1964); (5) dUMP hydroxymethylase (Roscoe and Tucker, 1966); (6) HMdUMP kinase (Kahan, 1971); and (7) DNA polymerase (Yehle and Ganesan, 1973).

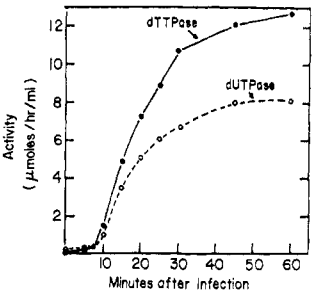

FIGURE 2: Induction of dTTPase–dUTPase. At the indicated times after phage infection, 2-ml samples of the culture were removed and chilled rapidly to 0°. Lysozyme was added directly to lyse the cells. The supernatant fluid from a 15 min, 10,000*g* centrifugation was assayed for dTTPase (●) and dUTPase (○) activity.

forming units per milliliter and were infected with a multiplicity of five phage per cell. After 25 min the infected cells were harvested by centrifugation at 0° and were resuspended in 1% of the original culture volume of buffer A: 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.7 mM HSEtOH. The cells were lysed by adding lysozyme (100 μg/ml), DNase (10 μg/ml), and RNase (10 μg/ml) and incubating at 37° for 30–60 min until the suspension visibly cleared. The supernatant fluid following centrifugation at 4° for 15 min at 10,000*g* was defined as the crude extract. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Enzyme Assays. The dTTPase–dUTPase reaction mixtures contained 120 mM Tris-HCl (pH 8.5), 250 mM HSEtOH, 50 mM $MgCl_2$, 2 mM [^{3}H]dTTP or [^{3}H]dUTP (0.125 μCi), and enzyme in a total volume of 100 μl. After incubation for 15 min at 37°, the reactions were terminated by the addition of 100 μl of 100 mM EDTA or by heating at 100° for 1 min. The product (tritium-labeled dTMP or dUMP) was separated from the substrate on Dowex AG-1-X8 columns and quantitated by liquid scintillation counting (Price and Fogt, 1973). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate/hr. The assay was linear with respect to both time and enzyme concentration up to at least 50% hydrolysis of the substrate.

Results

Induction of dTTPase–dUTPase. The induction of dTTPase–dUTPase activity by φe began between 7 and 10 min after the infection of *B. subtilis* 3610 at 37° (Figure 2). The enzyme activity continued to increase throughout the infection until at least 45 min after the addition of phage when phage-induced lysis of the cells began. The kinetics of enzyme induction were essentially identical for dTTPase and dUTPase, consistent with the hypothesis that one enzyme is responsible for both activities. Vigorous aeration of the cultures was necessary for maximum levels of enzyme induction. The specific activities of dTTPase in different crude extracts prepared 25 min after phage infection ranged from 25 to 90 units/mg of protein, and represented as much as a 100-fold increase over the level of activity in uninfected cell extracts. The relative increase in dUTPase activity to 20–70 units/mg of protein was less, about 20-fold, since uninfected *B. subtilis* extracts do possess a significant dUTPase activity (about 2 units/mg).

A number of control experiments were performed to prove that the induction of the dTTPase–dUTPase was specifically the result of φe infection. First, to rule out possible induction by a low molecular weight compound in the phage lysate, a "mock" infection was carried out with the supernatant fluid from a φe lysate centrifuged at 25,000*g* for 1 hr to sediment greater than 99% of the phage. Second, to eliminate possible induction by defective phage, a culture of *B. subtilis* 3610 was treated with 3 μg of mitomycin C/ml (Trilling and Aposhian, 1965). In both experiments, the level of dTTPase–dUTPase activity was no higher than in extracts of untreated uninfected *B. subtilis*.

Furthermore, the addition of 100 μg of chloramphenicol/ml, 10 μg of actinomycin D/ml, or 20 μg of rifampicin/ml at the time of phage infection completely prevented the induction of dTTPase–dUTPase. Chloramphenicol, added at various times after infection, shut off enzyme induction almost immediately; the effects of actinomycin D and rifampicin were slower. Thus, the induction of dTTPase–dUTPase is dependent upon protein and RNA synthesis, suggesting that at least a part of the enzyme is made *de novo* after phage infection.

Size of dTTPase–dUTPase in Crude Extracts. The φe-induced dTTPase–dUTPase activity appears to be a membrane-associated enzyme; 85–95% of the activity in a crude extract was pelleted by centrifugation at 100,000*g* for 1 hr. The enzyme in the crude extract was heterogeneous in size as shown by sucrose density gradient centrifugation or agarose gel filtration chromatography (average particle weight estimated to be larger than 5×10^7 but smaller than 1.5×10^8 daltons). Under conditions of very gentle lysis (Price and Cook, 1972), 55–90% of the enzyme could be pelleted with large membrane fragments at 10,000*g*. However, vigorous sonic oscillation of crude extracts could partially prevent the later pelleting of the enzyme at 100,000*g*. Furthermore, the low level (1–5% of normal) dTTPase–dUTPase activities induced by presumptive structural and regulatory gene mutants are similarly associated with the membrane (Dunham, 1973).

The appearance of the φe nucleotidohydrolase in the membrane fraction does not appear to be an artifact of the extract preparation methods employed. The enzyme remains active and pelletable at 100,000*g* even when the cells are lysed in the absence of HSEtOH. Furthermore, the enzyme does not adhere nonspecifically to membranes, since addition of solubilized enzyme (see below) to uninfected cells before lysis or to the 100,000*g* pellet from an uninfected extract does *not* cause the activity to become sedimentable at 100,000*g*. The dTTPase–dUTPase does have some unusual "adhesive" properties, however. It will quantitatively bind to Pharmacia

TABLE I: Purification of ϕe^+-Induced dTTPase–dUTPase.

Fraction	Volume (ml)	Total Activity (units)		Specific Activity (units/mg)		Purification Factor	dTTPase/dUTPase
		dTTPase	dUTPase	dTTPase	dUTPase		
10,000*g* supernatant fluid[a]	228	38,300	30,550	24.5	19.6	1	1.3
10,000*g* pellet[a]	20	910	550	2.2	1.3		1.7
100,000*g* supernatant fluid[b]	220	3,960	6,160	2.9	4.5		0.64
100,000*g* pellet[b]	13	25,220	20,670	104	86	4.2	1.2
Sodium deoxycholate supernatant fluid[c]	17	21,000	16,470	138	108	5.6	1.3
Pooled A 5m[d] fractions	210	17,220	13,650	270	193	11	1.4
A 5m peak tube	2	330	270	320	265	13	1.2
DEAE fraction I[e] (pooled fractions)	60	1,690	1,410	N.d.[f]	N.d.	N.d.	1.2
DEAE fraction I (peak tube)	7	560	475	150	125	6	1.2
DEAE fraction II[e] (pooled fractions)	100	3,050	2,540	N.d.	N.d.	N.d.	1.2
DEAE fraction II (peak tube)	7	510	435	370	310	15	1.2

[a] From 20 l. of infected cells. [b] From a 4-hr 100,000*g* centrifugation at 4° (27,000 rpm in the SW27 rotor of a Beckman L2-65B centrifuge; 38-ml buckets). [c] From a 90-min 100,000*g* centrifugation at 4° (40,000 rpm in a 40.3 rotor; 6.5-ml tubes) in the presence of 5% deoxycholate. [d] The sodium deoxycholate supernatant fluid was applied (in thirds) to a 50 × 2.5 cm column of Bio-Gel A 5m resin. The column was developed at room temperature with buffer A + 1% (v/v) Triton X-100; 2-ml fractions were collected. The fractions containing dTTPase–dUTPase activity were pooled. [e] The pooled A 5m fractions were applied to a 20 × 2.4 cm column of DEAE-cellulose. The column was developed at room temperature as described in the text. [f] N.d. = not determined.

Blue Dextran 2000 in the presence or absence of 1% Triton, although not in the presence of 2% sodium deoxycholate. In addition, solubilized enzyme binds to catalase in the absence (but not in the presence) of 1% Triton X-100 (Dunham, 1973).

Purification of dTTPase–dUTPase. The purification of the ϕe-induced dTTPase–dUTPase is outlined in Table I. The first 100,000*g* centrifugation separated the membrane-associated phage-induced enzyme from the *B. subtilis* dUTPase, which has an approximate molecular weight of 40,000 and remains in the supernatant fluid during a 100,000*g* centrifugation (Dunham, 1973).

The phage-induced enzyme was solubilized by making the resuspended 100,000*g* pellet 5% (w/v) in sodium deoxycholate by adding solid sodium deoxycholate and incubating for 0.5 hr at 4°. The solubilized enzyme was recovered in the supernatant fraction from a second 100,000*g* centrifugation. At this stage the enzyme was not truly solubilized, however, as it reaggregated to a high molecular weight form (that is, it eluted in the void volume of a Bio-Gel A 5m column) in the absence of detergent.

The dTTPase–dUTPase in the first 100,000*g* pellet could also be partially solubilized by 3 M urea or 1% (v/v) Triton. However, neither of these agents produced the degree of solubilization nor the recovery of activity obtained with 5% sodium deoxycholate. Likewise, lower concentrations of sodium deoxycholate left residual dTTPase–dUTPase activity in the second 100,000*g* pellet. Since sodium deoxycholate solutions tend to form gels, even at ambient temperatures and especially in the presence of NaCl, the second 100,000*g* supernatant fluid was subjected to Bio-Gel A 5m gel filtration chromatography to exchange 1% Triton for sodium deoxycholate. The degree of solubilization achieved by 5% sodium deoxycholate was maintained by 1% Triton.

Further purification was attempted by DEAE-cellulose column chromatography using stepwise elution. After application of the Bio-Gel pooled fractions, the column was washed with buffer A. Two peaks of enzymatic activity were found, one eluting with buffer A + 1.0 M NaCl (fraction I) and the other with buffer A + 1.0 M NaCl + 1% Triton (fraction II).

Gel filtration chromatography in the presence of 1% Triton (Figure 3a) revealed that dTTPase–dUTPase from DEAE fractions I and II (and the sodium deoxycholate supernatant fraction) displayed the same molecular weight, 100,000. Gel filtration chromatography in the absence of detergent suggested that enzyme in fraction I may be "more nearly solubilized" than that in fraction II, since some of the activity in fraction I remained as a low molecular weight (100,000) form (Figure 3b). It is not known whether the high molecular weight (430,000) form seen in the absence of detergent (Figure 3b) represents self-aggregation of the low molecular weight species to form a tetramer, or aggregation with extraneous membrane components. However, both DEAE fractions are solubilized and not pelletable at 100,000*g*.

The ratio of dTTPase to dUTPase activity remained constant throughout the purification, again suggesting that the dTTPase and dUTPase activities reside in a single enzyme. Enzyme eluting in DEAE fraction I (without detergent) was used for the remainder of the experiments, except where noted. DEAE fraction I can be stored at −16° in 50% glycerol and DEAE fraction II at 4° for at least 5 months without loss of activity.

Dependence of Activity on HSEtOH and $MgCl_2$. Both dTTPase and dUTPase activities showed an absolute requirement for a sulfhydryl compound which could be fulfilled by HSEtOH, monothioglycerol, or dithiothreitol. [It is of interest that dTTPase and dUTPase activities are not dependent upon HSEtOH when assayed in crude extracts prepared in the presence or absence of HSEtOH (Dunham, 1973)]. The optimum concentration of HSEtOH for both activities (in DEAE fraction I) was 250 mM (Figure 4a). The requirement for a sulfhydryl compound was confirmed by the demonstration that dTTPase and dUTPase activities (at 0.25 mM HSEtOH) were inhibited in parallel by increasing concentrations of HgBzOH (Figure 4b). Higher concentrations of HSEtOH

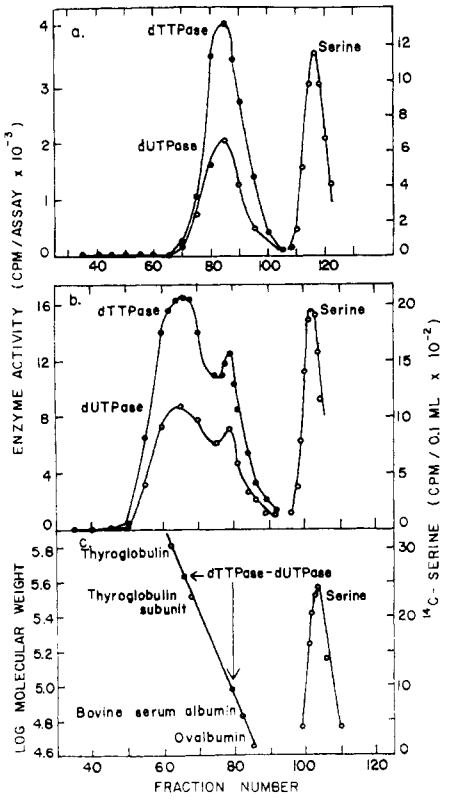

FIGURE 3: Molecular weight determination of dTTPase–dUTPase from DEAE fraction I. The following samples were applied to a 49 × 2.5 cm column of Bio-Gel A 5m resin: (a) 1 ml of DEAE fraction I and 0.2 μCi of [^{14}C]serine; (b) 1.5 ml of DEAE fraction I and 0.2 μCi of [^{14}C]serine; and (c) 5 mg of thyroglobulin (mol wt 640,000), 5 mg of ovalbumin (mol wt 45,000), 5 mg of bovine serum albumin (mol wt 67,000), and 0.3 μCi of [^{14}C]serine. Column a was developed with buffer A containing 1% (v/v) Triton X-100; columns b and c were developed with buffer A alone. All three columns were developed at room temperature; 1.9-ml fractions were collected from column a; 2.5-ml fractions were collected from columns b and c. The dTTPase (●) and dUTPase (○) activities were measured on 70-μl samples from fractions from columns a and b; the time of incubation at 37° was increased to 60 min. The standard proteins applied to column c were located by measuring the absorbance of the fractions at 280 nm. The dTTPase and dUTPase activities eluted with a constant ratio of 2:1 in a peak whose width was identical with the width of the albumin standard peak.

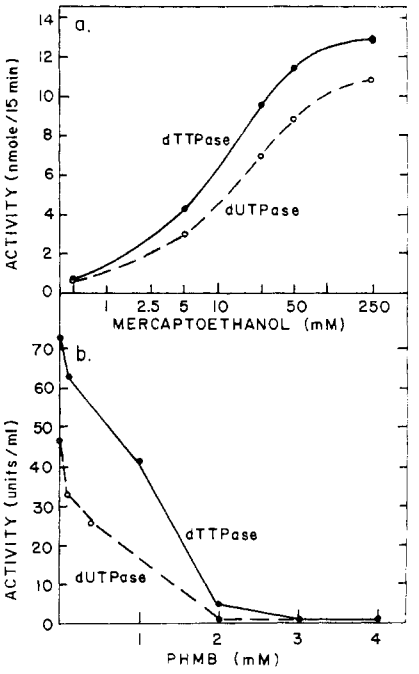

FIGURE 4: Sulfhydryl requirement of partially purified dTTPase–dUTPase. (a) Dependence of enzyme activity on HSEtOH (log scale). The standard incubation mixture was modified to contain 2.5 mM $MgCl_2$ and the indicated concentrations of HSEtOH; 2 μl of DEAE fraction I was used for each assay. (b) Inhibiton by HgBzOH (linear scale). The standard incubation mixture was modified to contain 0.25 mM MSH and the indicated concentrations of HgBzOH; 10 μl of DEAE fraction I was used for each assay.

(25 mM) totally protected the enzyme from inactivation by 1 mM HgBzOH.

Both dTTPase and dUTPase activities displayed an optimum $MgCl_2$ concentration of 50 mM. In the absence of added $MgCl_2$, the activities were 1 and 25% of the maximal levels for dTTPase and dUTPase, respectively; both activities were completely inhibited by the addition of EDTA (but were unaffected by fluoride ions). As substitutes for $MgCl_2$, $MnCl_2$ and $ZnCl_2$ functioned well; $CaCl_2$, $BaCl_2$, NaCl, and KCl were weak activators; and $CoCl_2$, $NiCl_2$, and $HgCl_2$ were ineffective (Dunham, 1973).

In general, the other metal salts were better able to substitute for $MgCl_2$ in the dUTPase assay than in the dTTPase assay. This observation, along with the disparity in dTTPase and dUTPase activities seen when no $MgCl_2$ is added to the assay, can perhaps be explained in terms of the 35-fold difference in K_M's for the two substrates (see below). The actual substrates may be triphosphate–cation complexes. Low concentrations of salts (added inadvertently in other reaction components) or salts which form complexes poorly may still yield effective substrate concentrations in the range of the K_M for dUTP (11 μM), but 10- to 100-fold lower than the K_M for dTTP (400 μM).

The enzyme was stimulated by the addition of NaCl to the standard assay mixture. The optimum stimulation of dTTPase given by NaCl (at 0.2 M) was 1.3-fold; the optimum stimulation of dUTPase by NaCl (at 0.7 M) was 2.3-fold. The differential degree of stimulation of dTTPase and dUTPase activities by NaCl may be another manifestation of the large difference in K_M for the two substrates. KCl stimulated dTTPase–dUTPase activity to a slightly greater extent and LiCl to a slightly lesser extent than NaCl. The ϕe-induced dTTPase and dUTPase exhibited parallel activities with several buffers over a broad range of pH from 7.0 to 10.5 (Dunham, 1973).

Kinetic Constants. The K_M's for dTTPase and dUTPase activities in both DEAE fractions I and II were 400 μM for dTTP and 11 μM for dUTP (Figure 5). Furthermore, the K_M's for dTTP and dUTP of the partially purified enzyme were essentially identical with those of the original 100,000*g* pellet fraction. Thus, the gross kinetic properties of this enzyme, as reflected by K_M values, appear to be unaffected by the solubilization procedures to which the enzyme was subjected.

When higher substrate concentrations were used in the K_M determination for dUTP, a biphasic double reciprocal plot was obtained, with a break at 0.1 mM substrate and a second K_M of approximately 70 μM (obtained from the 1/dUTP intercept). This effect was seen in the first 100,000*g* pellet fraction and DEAE fractions I and II; thus, it was not an artifact resulting from solubilization of the enzyme. Similar kinetic data for dUTP were obtained with the enzymes induced by two ϕe mutants which were defective in dTTPase–dUTPase synthesis (Dunham, 1973). Such a biphasic plot was not obtained for dTTP, even with substrate concentrations ranging from 2.3 mM to 2.2 μM (Dunham, 1973).

The K_i's for dTTP and dUTP were measured in order to determine which of the dUTPases, the high or low K_M enzyme, corresponds to the dTTPase active site. With dTTP as substrate and dUTP as inhibitor, inhibition was found to be of the competitive type, with a K_i of 14 μM (Figure 5a). Likewise, using dUTP as substrate and dTTP as inhibitor, inhibition was also competitive with a K_i of 700 μM (Figure 5b). Note that the K_i for each substrate was approximately equal to its K_M, strongly suggesting that the ϕe-induced dTTPase–dUTPase is a single enzyme with one active site for the hydrolysis of its two substrates, dTTP and dUTP, whose K_M's are 400 and 11 μM, respectively.

The high K_M dUTPase activity is probably of viral origin since the residual amount of dUTPase activity in the pellet from a 100,000g centrifugation of a crude extract of uninfected *B. subtilis* can account for no more than 1–2% of the activity associated with the high K_M dUTPase seen in infected extracts. However, we have been unable to determine whether the high K_M dUTPase is an altered conformation, the ϕe-induced low K_M dUTPase.

Inhibition constants were also determined for a number of other nucleotides and possible end product inhibitors of dTTPase and dUTPase (Table II). [We have demonstrated that hydrolysis of dTTP and dUTP leads to stoichiometric amounts of dTMP or dUMP and PP_i (Dunham, 1973)]. In each case, the inhibition was competitive (Dunham, 1973) and the K_i's determined with respect to both substrates were approximately equal.

Substrate Specificity. The partially purified enzyme has a strong preference for dTTP or dUTP as substrate with a dTTPase–dUTPase V_{max} ratio of 1.3. Less than 0.5% of the activity with dTTP was seen with dATP, dGTP, dCTP, dUDP, dTDP, UTP, thymidine 5′-tetraphosphate, or β,γ-methylene-dTTP (Dunham, 1973). The only substrate hydrolyzed at a detectable rate other than dTTP and dUTP was 4-thio-dTTP (48% of the rate with dTTP). Thus, the active site of the enzyme must have at least three recognition sites: one for the thymine or uracil ring structure, a second for the 2′ position on the deoxyribose ring, and a third for the γ-phosphate group.

The specificity of the active site for dUTP is reflected by the fact that the K_i's for the other deoxyribonucleoside triphosphates (Table II) are at least 30-fold higher than the K_i for dUTP in the special case of dTTP and 600-fold higher in the cases of dCTP, dATP, and dGTP. The low K_i value for dUDP implies that the inability of dUDP to act as a substrate probably does not lie in its inability to bind to the enzyme.

TABLE II: Competitive Inhibition Constants.

Inhibitor	K_i (mM) dTTPase	K_i (mM) dUTPase
dTTP		0.70
dTDP	0.67	1.0
dTMP	7.4	2.6
dUTP	0.014	
dUDP	0.046	0.037
dUMP	1.2	0.96
UTP	8.6	6.8
dCTP	11	18
dATP	11	7.0
dGTP	40	8.7
PP_i	0.27	0.45
P_i	31	56

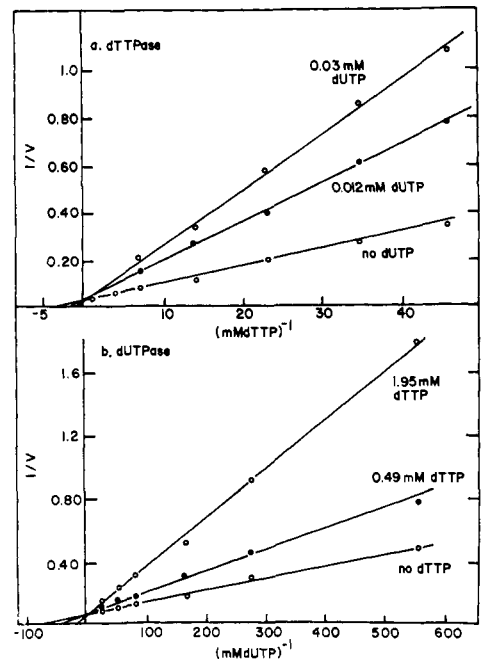

FIGURE 5: K_M and K_i determinations for DEAE fraction I. All enzyme assays were performed in duplicate with concentrations of enzyme low enough to ensure that less than 10% of the substrate was hydrolyzed. Velocities are expressed as units of enzymatic activity/ml of enzyme: (a) [^{3}H]dTTP as substrate and the indicated concentrations of dUTP as inhibitor; (b) [^{3}H]dUTP as substrate and the indicated concentrations of dTTP as inhibitor.

Discussion

The ϕe-induced dTTPase–dUTPase appears to be a single protein capable of hydrolyzing dTTP and dUTP to their respective monophosphates. Both activities are induced in parallel after phage infection; the ratio between the two activities remains constant during purification. The dTTPase and dUTPase exhibit the same Mg^{2+}, pH, and HSEtOH optima; both activities are inhibited in parallel by HgBzOH. Kinetic analysis shows that a single active site on the enzyme catalyzes the hydrolysis of both dTTP and dUTP. Furthermore, mutants of ϕe defective in the induction of dTTPase are also defective in the induction of dUTPase (Roscoe, 1969a,b; Dunham, 1973).

The ϕe-induced dTTPase–dUTPase is unique among all known T-even or *B. subtilis* phage-induced enzymes involved in nucleotide metabolism in that it is membrane associated. The dTTPase induced by SP82G, another HMU–DNA phage (Price *et al.*, 1972), also is sedimentible under our conditions (Dunham, 1973). Several *Bacillus* enzymes are known to be membrane associated, including NADH oxidase (Yu and Wolin, 1972), ATPase (Mirsky and Barlow, 1973), phospholipase A_1 (Kent and Lennarz, 1972), and alkaline phosphatase (Wood and Tristam, 1970). However, these host enzymes were solubilized under much gentler conditions than those required for solubilization of the ϕe nucleotidohydrolase. Although the high specific activity of dTTPase–dUTPase in crude extracts [50 to 600 times higher than those of the other ϕe-induced enzymes (Roscoe and Tucker, 1966)] could be due to a high turnover number for dTTPase–dUTPase, it is also possible that the enzyme has a structural function in a membrane-associated DNA-synthesizing complex in addition to its

catalytic function. The existence of such a complex is supported by the observation of Kolenbrander *et al.* (1973) that phage SP82 HMU–DNA becomes membrane associated after infection of *B. subtilis*. Also, Levner and Cozzarelli (1972) have demonstrated that formation of a rapidly sedimenting DNA–protein complex is the first step in the processing of newly synthesized SP01 phage HMU–DNA.

In view of the 35-fold difference in the K_M values for dTTP (400 μM) and dUTP (11 μM), it may be more appropriate to refer to the ϕe-induced dTTPase–dUTPase as a dUTPase first and a dTTPase second. The most likely function of this enzyme (in view of its narrow substrate specificity) is to hydrolyze dUTP to prevent the incorporation of uracil into phage DNA and at the same time to produce dUMP, the substrate for the ϕe-induced dUMP hydroxymethylase. If so, its function would be analogous to that of the T4 phage-induced dCTPase–dUTPase, whose dCTPase activity prevents the incorporation of cytosine from dCTP into T4 DNA and simultaneously produces dCMP, the substrate for the T4-induced dCMP hydroxymethylase (Warner and Hobbs, 1968; Price and Warner, 1969).

Unlike the ϕe-induced dTTPase–dUTPase, the T4-induced dCTPase–dUTPase (Price and Warner, 1969) is found in the cytoplasm of T4-infected cells and is not membrane bound. However, the T4 enzyme is activated by mercaptoethanol, requires Mg^{2+} for activity, and has a pH optimum of 8.5, similar to the ϕe enzyme. The T4 dCTPase–dUTPase is inhibited by potassium fluoride unlike the ϕe dTTPase–dUTPase. The T4-induced enzyme hydrolyzes dCDP and dUDP as well as dCTP and dUTP, while the ϕe enzyme hydrolyzes only dTTP and dUTP. The K_M for the T4 dCTPase is 6 μM, and the K_M for the T2 dUTPase is 3.3 μM (Greenberg, 1966). Amber mutants of T4 with essentially no dCTPase–dUTPase activity make only small amounts of DNA (Warner and Hobbs, 1967), since cytosine-containing DNA is rapidly degraded by phage-coded nucleases (Price and Warner, 1969).

On the other hand, ϕe mutants inducing only 1–5% of normal dTTPase–dUTPase activity are viable. When grown in a thymine-requiring host, the phage incorporate some thymine, but no uracil into the mutant DNAs (Roscoe, 1969b; Dunham, 1973). Perhaps the dTTPase–dUTPase appears to be nonessential because there exist other bacterial and viral proteins (Figure 1) which affect deoxyuridine and deoxythymidine levels: dUTPase, dCMP deaminase, dTMP synthetase inhibitor, etc. Thus a more complete understanding of the role of dTTPase–dUTPase in ϕe infection with respect to preventing thymine and/or uracil incorporation into phage DNA or supplying dUMP as precursor to HMdUMP must await isolation and characterization of additional host and phage mutants.

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