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A Homogeneous, Thermostable Deoxythymidine Kinase from *Bacillus stearothermophilus*†

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ABSTRACT: A deoxythymidine kinase has been purified from *Bacillus stearothermophilus* 16,000-fold over the activity of the extract. The molecular weight of the kinase determined by molecular sieve chromatography (0.005 M Tris–glycine (pH 8.3), 4° was found to be 52,000. A molecular weight estimation by polyacrylamide gel electrophoresis (10% acrylamide, pH 10.5) revealed isomers of molecular weight 54,000 and 108,000. A polypeptide chain analysis revealed a single protein band of molecular weight 28,500. The Michaelis–Menten constant (K_m) for deoxythymidine was $4.8 \pm 0.8 \times 10^{-5}$ M at 75° and $5.3 \pm 0.6 \times 10^{-7}$ M at 37°. The K_m for Mg-ATP^{2-} was $4.3 \pm 0.2 \times 10^{-4}$ M at 75° and remained constant at $3.8 \pm 0.4 \times 10^{-4}$ M at 37°. Deoxythymidine triphosphate (dTTP) functioned as a negative effector. The inhibition constant (K_i) for dTTP was 23×10^{-6} M at 75° and 9.3×10^{-6} M at 39°. Exposure of the enzyme at 75° for 60 min resulted in no loss of activity. When exposed at 100° for 30 min, a 40% loss of activity was observed. The catalytic power of the kinase increased from 1.25 μmol of dTMP hr^{-1} μg^{-1} at 32° to 220 μmol at 90°.

The role of deoxythymidine kinase in regulation of DNA synthesis is yet to be established, however, an increased activity of the enzyme has always been reported preceding or concomitant with the onset of DNA synthesis. The phenomenon has been observed in a variety of extracts from plants (Hotta and Stern, 1963; Wanka *et al.*, 1964), regenerating rat liver (Bollum and Potter, 1959; Bresnick *et al.*, 1970), virus infected cells (McAuslan, 1963; Hatanaka and Dulbecco, 1967; Kit *et al.*, 1970), adrenal gland stimulated by ACTH, insect epithelium stimulated by ecdysone (Masui and Garren, 1970; Brookes and Williams, 1965), and mammalian cells during the S phase (Stubblefield and Mueller, 1965). The enzyme is also very active in mammalian tumors (Sneider *et al.*, 1969; Bresnick and Thompson, 1965). In general, the activity of the enzyme appears to be very responsive to stimuli which ultimately result in DNA synthesis.

So far, only partial purification of this enzyme has been achieved due to its instability. The method of preparation of

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deoxythymidine kinase from *Escherichia coli* (Okazaki and Kornberg, 1964a) yields an enzyme with the highest specific activity reported, but it has the drawback of requiring serum albumin for the measurement of activity. Such a requirement renders the study of some of the properties of kinase difficult or impossible. We report here a method for preparing the enzyme from extracts of *Bacillus stearothermophilus* in an apparent homogeneous state and a study of a few interesting properties of the macromolecule. The thermostable kinase closely resembles its counterpart derived from mammalian organs (calf thymus) as far as affinities for substrates, regulators, and molecular weight.

Materials and Methods

The labeled ^{14}C nucleosides and nucleotides were purchased from Amersham/Searle Corp. and New England Nuclear; nucleosides, nucleotides, lactate dehydrogenase, catalase, hexokinase, pepsinogen, and alcohol dehydrogenase were from P-L Biochemicals; soybean trypsin inhibitor and chicken egg-white lysozyme were from Worthington; streptomycin sulfate was from E. R. Squibb and Sons; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] was from CalBiochem; bovine serum albumin was from Pentex; sodium dodecyl sulfate was from Fisher. Pyruvate kinase, tris(hydroxymethylaminomethane), ammonium sulfate (ultra pure), apoferritin, and sperm whale myoglobin were products of Mann. Sodium phosphoenol pyruvate was purchased from Sigma; pepsin from Nutritional Biochemical Co.; Sephadex G-200 from Pharmacia; DEAE-cellulose paper (DE 81), microgranular DEAE-cellulose powder DE 52, and cellulose phosphate P11 from Whatman. *Bacillus stearothermophilus* strain 10 was originally supplied by Dr. L. L. Campbell.

Assay of the Enzyme. Two types of enzymatic assay (Orengo, 1969) were used. The radiochemical assay measures the conversion of nucleosides to nucleotides by chromatographic separation of the reactants and products on DEAE-cellulose paper. The optical assay measures the oxidation of NADH. In the latter assay, pyruvate kinase is coupled with lactate dehydrogenase in order to measure ADP. The disappearance of NADH, measured by a decrease in absorbance at 340 nm, is a measure of the kinase activity.

Analytical Disc Gel Electrophoresis. A Buchler analytical polyacrylamide vertical disc gel electrophoresis apparatus was used. The gels were made in glass tubes (0.5 × 8 cm), and a constant current of 2.5 mA/gel was applied for 2–5 hr at 5°. Two buffer systems, the Tris–glycine system (Ornstein, 1964; Davis, 1964; Maurer, 1971) and the Tris–boric acid–Na_2EDTA system, were used.

The running pH was 8.3 in the concentration gel and 9.5 in the separation gel for the Tris–glycine system; 8.28 for the Tris–boric acid–Na_2EDTA system. The latter was used without concentration gel and the gel buffer was prepared with 4.31 g of Tris, 2.20 g of boric acid, and 0.37 g of Na_2EDTA for every 100 ml. One volume of the buffer was mixed with 1 volume of ammonium persulfate and 2 volumes of *N,N'*-methylenebisacrylamide–acrylamide solutions. The buffer of the electrode chambers was a 1:4 dilution of the gel buffer (booklet of instruction on the use of the Buchler electrophoretic apparatus, 1971).

The gels were fixed in 12.5% Cl_3CCOOH (w/v) for 15–30 min at room temperature, then stained for either 30–60 min at 65° or overnight at room temperature. The staining solution was prepared by mixing 100 ml of 0.2% Coomassie Brilliant Blue, 100 ml of absolute ethanol, and 22 ml of glacial acetic acid. Destaining was carried out in a solution containing 250 ml of absolute ethanol and 100 ml of glacial acetic acid per liter of water. Stained gels were read in a Gilford spectrophotometer Model 240 equipped with a gel scanner attachment. Duplicate gels were cut in half, lengthwise. One-half was fixed and stained as described above, while the other half was frozen on Dry Ice and sectioned in 1-mm thick slices. The slices were placed in 0.1 M Tris-Cl (pH 7.6) for 1 hr and the enzyme activity was determined by either the radioactive or optical assay.

Estimation of Molecular Weight by Molecular Sieve Chromatography (Sephadex). An aliquot of fraction VII (see Table I) was applied to a Sephadex G-200 column (2.54 × 95.25 cm) which had been equilibrated with 0.05 M Tris–glycine buffer (pH 8.3). The same buffer was used to elute the enzyme at a rate of 8.7 ml/hr. Fractions of 2.175 ml were collected and read at 280 nm, and their kinase activity was measured by the radioactive assay. The column was previously calibrated with the following molecular markers: catalase from beef liver (mol wt 244,000), lactate dehydrogenase from chicken heart (142,000), hexokinase from yeast (102,000), alcohol dehydrogenase from horse liver (84,000), and bovine serum albumin (mol wt 66,000). Blue dextran and DPN-alanine were used to measure V_0 and $V_0 + V_i$. K_{av} was a function of molecular weight according to

$$K_{av} = 2.3805 - 0.3947 \log \text{mol wt}$$

Estimation of Molecular Weight by Polyacrylamide Gel Electrophoresis—Ferguson Relationship (Ferguson, 1964). Fraction VII was used. The preparation of gels, electrophoresis, and measurements of mobility were carried out as described by Rodbard and Chrambach (1971). The multiphasic buffer system B was used. The separation pH was 10.20 and the electrophoresis was carried out at 24°. The calibration curve was obtained from the following proteins: soybean trypsin inhibitor (mol wt 22,700), swine stomach mucosa pepsinogen (40,400) pepsin (dimer) (71,000), beef liver catalase (244,000), and horse apoferritin (480,000). The total gel concentrations varied from 6 to 12%. Stained gels were read in a Gilford spectrophotometer Model 240 equipped with a gel scanner attachment. X was found to be a function of the molecular weight according to the following linear equation: mol wt = $(-8.93 + 2.62X) \times 10^4 \pm 4000$, where X is the slope of a plot of 100 log ($R_m \times 100$) *vs.* percentages of acrylamide and R_m is equal to distance to the zone/distance to the indicator dye.

Estimation of the Molecular Weight of the Polypeptide Chains by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Fraction VII was used. The preparation of gels and electrophoresis were carried out according to Laemmli (1970). The total gel concentration was 15%. The calibration curve was obtained from the following proteins: bovine serum albumin (mol wt 66,000), ovalbumin (45,500), sperm whale myoglobin (17,800), and chicken egg-white lysozyme (14,400). Molecular weight was calculated from the following equation: log mol wt = $5.134 - 1.205R_m \pm 9\%$ (with a 95% confidence).

Steady-State Kinetics and Treatment of Data. Initial velocities were measured. In all cases the conversion of substrate to product was maintained below 5% by varying the time of incubation. The Michaelis–Menten constants were

[1] Abbreviations used are: dTTP, deoxythymidine triphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I: Purification of Deoxythymidine Kinase from *B. stearothermophilus*.[a]

Fractions and Steps	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Purification units/mg of protein
I. Extract	0.024	96.9	7.0	0.0034
II. Streptomycin	0.032	158.9	2.5	0.013
III. Ammonium sulfate	0.129	116.4	(1.5)	0.086
IV. Ammonium sulfate	1.118	86.1	(4.56)	0.245
V. Heat precipitation	1.796	129.3	(0.23)	7.741
VI. Cellulose phosphate	0.625	90.0	0.057	10.965
VII. DEAE-Cellulose	5.333	70.4	0.097	54.979

[a] The radiochemical assay was used and the activity measured at 37°. The values in parentheses were measured by uv absorbance using the following formula: $A_{280} \times 1.45 - A_{260} \times 0.74$ = mg of protein/ml. All the other values were obtained by the procedure of Lowry *et al.*, 1951. The procedure has been repeated and the data presented are representative.

obtained by applying a weighted least-squares analysis of the data (Wilkinson, 1961). The inhibitory constants (K_i) were obtained by graphic analysis (Dixon, 1953). The estimated uncertainty in the kinetic parameters is about ±10%.

The concentrations of nucleosides and nucleotides were determined spectrophotometrically in 10^{-2} M HCl, using the published molar extinction coefficients. Protein was measured with Folin phenol reagent (Lowry *et al.*, 1951).

Results

Purification of the Enzyme.

Growth of Bacterial Cells. B. stearothermophilus strain 10 was grown in a trypticase medium (Saunders and Campbell, 1966) at 60° in a 250-l. fermentor. The cells were harvested by continuous flow centrifugation and stored at −20°.

Preparation of the Bacterial Extract. Frozen cells (500 g) were suspended in 0.05 M Tris-Cl (pH 7.51), 0.5 g of packed cells/ml of buffer. Aliquots of 100 ml were disrupted with a Branson Sonifier Model W185 by five 1-min bursts at 70 W. The temperature was kept below 10°. Cell debris was removed by centrifugation at 12,000*g* for 30 min. A 10% deoxycholate solution was added to yield a final concentration of 0.2%. The preparation was then centrifuged at 123,000*g* for 2 hr. Protein concentration, usually around 20 mg/ml, was adjusted to 7.0 mg/ml with distilled water (fraction I).

Streptomycin Treatment. Streptomycin (5%, 250 ml) was added with continuous stirring to 1 l. of fraction I. After 1 hr of standing in the cold room, the suspension was centrifuged for 20 min at 20,000*g*. The supernatant yielded fraction II.

Ammonium Sulfate Treatment. Fraction II was brought to 35% saturation by the slow addition of ammonium sulfate with continuous stirring. After 1 hr of standing in the cold room, the suspension was centrifuged at 20,000*g* for 20 min. The precipitate was dissolved in 0.01 M Tris-Cl (pH 7.6, 60 ml of Tris/l. of fraction II). The precipitate should be dissolved as soon as possible. It may be necessary to stir the suspension in the cold overnight in order to achieve maximal recovery. Protein concentration was adjusted to 1.5 mg/ml (fraction III). To a liter of fraction III, 108.5 g of ammonium sulfate was added slowly with continuous stirring. After 30 min of standing in the cold room, the suspension was centrifuged at 20,000*g* for 10 min. The precipitate was dissolved in a minimum volume of 0.01 M Tris-Cl (pH 7.6) (fraction IV). At times, an overnight, gentle stirring in the cold was necessary to increase the yield of the enzyme.

Heat Treatment. Fraction IV was heated in a water bath to 75° for 1 hr. The precipitated proteins were removed by centrifugation at 20,000*g* for 20 min. This step produced an increase of 50% in the total activity. A possible explanation is that an inhibitor was removed by this treatment. The supernatant constituted fraction V.

Cellulose Phosphate Treatment. Portions of fraction V (25 ml) were charged on a Sephadex G-25 column (2.5 × 73 cm), previously equilibrated with 0.01 M Tris-Cl–10% glycerol (pH 7.25). Fractions of 4 ml were collected and the void volume was pooled. The void volumes of three consecutive runs were combined and charged on a cellulose phosphate column (2.5 cm × 8 cm), previously equilibrated with 0.01 M Tris-Cl–10% glycerol (pH 7.25). The flow rate was 1 drop/5 sec and fractions of 7.2 ml were collected. The enzyme was not retained by the resin. The active fractions were pooled and yielded fraction VI.

DEAE-Cellulose Chromatography. DEAE-cellulose was washed with 0.1 M EDTA (pH 7.0) and then equilibrated with 0.1 M Tris-Cl–10% glycerol until the pH and conductance of the effluent buffer were constant. A portion of fraction VI (8 mg of protein) was charged on the column. The column was washed with 115 ml of 0.01 M Tris-Cl–10% glycerol, (pH 7.24), and then with 250 ml of 0.1 M Tris-Cl–10% glycerol (pH 7.24). The enzyme was eluted with 0.1 M Tris-Cl–10% glycerol–0.03 M ammonium sulfate (pH 7.24). Subsequent elutions at a higher concentration of ammonium sulfate (0.05, 0.1, and 0.3 M) did not produce any active fraction. The fractions were monitored at 280 nm to follow the protein elution. The enzymic activity was determined by the spectrophotometric assay. The fractions with the highest specific activity were pooled and dialyzed overnight against 4 l. of 0.01 M Tris-Cl–10% glycerol (pH 7.25). The dialysis buffer was changed once. To concentrate the enzyme, the dialyzed DEAE pool was charged on a DEAE-cellulose column (2.5 × 2 cm), equilibrated as above. The column was washed with 10 ml of the equilibration buffer. The enzyme was eluted with 0.01 M Tris-Cl–10% glycerol–0.2 M ammonium sulfate. The enzyme was dialyzed for 18 hr against a total of 4 l. of 0.01 M Tris-Cl–10% glycerol (pH 7.24). The buffer was changed after the first 8 hr. This constituted fraction VII.

Purity, Molecular Weight, and Polypeptide Chains Composition of the Enzyme. The specific activity of fraction VII is 16,000 times higher than that of the extract. The heat treatment appears to be the most efficient step in the purification procedure (Table I). Multiple electrophoretic separations were carried out at different concentrations of protein and acrylamide in order to study the homogeneity of the preparation. Two protein bands can be separated by electrophoresis in 10% polyacrylamide gels. This heterogeneity appears to be due to an interaction during the migration because the properties of the two protein bands depend on the concentration of acrylamide and the amount of protein charged per gel. In Figure 1, protein and activity

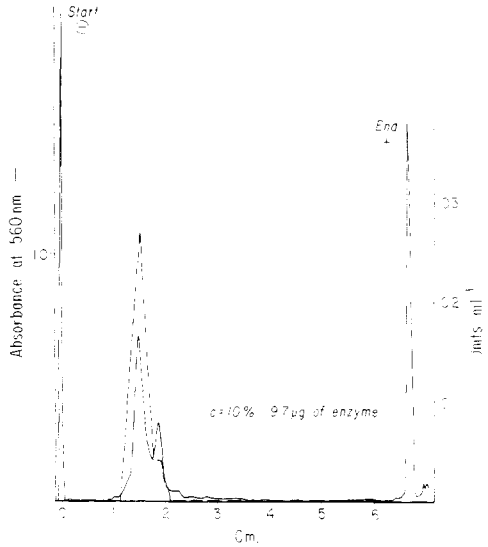

FIGURE 1: Protein and activity profile of deoxythymidine kinase. Fraction VII was used. The Tris-boric acid-Na_2EDTA buffer system was employed. The running pH was 8.28. No stacking gel was present. After electrophoresis the gel was sliced lengthwise. One-half was stained for protein with Coomassie Blue. The other half was frozen and cut into 1-mm thick sections. Each section was placed in 0.01 M Tris-Cl (pH 7.6) at 4°, for at least 1 hr. The enzymatic activity was measured by radiochemical assay.

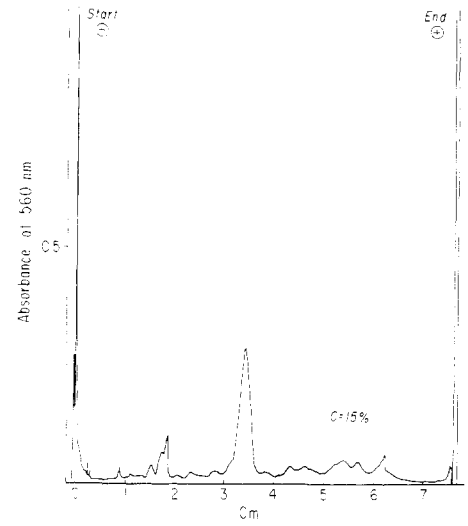

FIGURE 2: Protein profile of the polypeptide chains by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fraction VII was used. The preparation of gels, electrophoresis, and measurements of mobility were carried out as described in Materials and Methods.

profiles are compared in a separation carried out in the borate buffer system at a concentration of 10% acrylamide. The borate system was preferred to the Tris-glycine system because of its lower running pH (8.28), so that a better recovery of activity could be obtained from the gel slices. Activity is present in both protein bands, although the specific activity is higher in the region of the slow migrating band.

An estimation of the molecular weight of the enzyme was obtained by two independent methods.

Molecular sieve chromatography on Sephadex G-200 revealed one peak of activity corresponding to a molecular weight of 52,000.

Polyacrylamide gel electrophoresis was used as described in the Materials and Methods section. The gels revealed two size isomers having molecular weights of 108,000 and 54,000. The latter value is in good agreement with the 52,000 obtained from Sephadex G-200. The weak interactions and disulfide bridges among polypeptide chains were dispersed by the use of urea, 2-mercaptoethanol, and sodium dodecyl sulfate and the resultant product was electrophoresed on polyacrylamide gel (15%) in presence of sodium dodecyl sulfate (Laemmli, 1970). Figure 2 shows the composition of the product. Almost all of the protein is recovered as a polypeptide chain of 28,500. The other significant component of the electropherogram has a molecular weight of 54,000 and may be the undissociated kinase.

Phosphate Donor and Acceptor Specificity and Steady-State Kinetics. The eight commonly occurring nucleosides were tested as phosphate acceptors using ATP as phosphate donor. Deoxyuridine (dUrd) and deoxythymidine (dThd) were the only nucleosides capable of functioning as a substrate for the kinase reaction; dUrd (1.16 μmol of ADP formed) was as good as dThd (1.08 μmol). The others gave the following results: Urd, 0.04 μmol; Cyd, 0.03 μmol; dCyd, 0.06 μmol; Ado, 0.03 μmol; dAdo, 0.00 μmol; Guo, 0.00 μmol, and dGuo, 0.00 μmol. The concentration of all of the nucleosides was 1.2 mM.

When the specificity of the phosphate donor was tested, ATP and dATP were the most efficient substrates. The dThd concentration was 0.83 mM. A scale of efficiency of the phosphate donors could be arranged as follows: ATP (13.7 μmol of dTMP formed), dATP (12.3), UTP (5.1), ITP (4.2), XTP (4.1), GTP (3.1), CTP (3.0), dCTP (2.6), dUTP (2.3), dGTP (1.3), dTTP (1.0), AMP (0.6). The concentration of all of the nucleoside triphosphates and AMP was 2.37 mM. AMP was tested as phosphate donor to exclude the possibility that the nucleoside was phosphorylated by a nucleoside phosphotransferase. Brawerman and Chargaff (1954) have described a group of enzymes which catalyze the transfer of organically esterified phosphoric acid to nucleosides. These enzymes utilize a variety of low energy phosphates as donors.

The activity of the kinase depends strictly on Mg^{2+}. The concentrations of Mg^{2+} used were selected on the basis of the stability constants of $MgATP^{2-}$ and $MgADP^{-}$ measured by O'Sullivan and Perrin (1964).

dThd and $MgATP^{2-}$ were used to measure the kinetic parameters of the enzyme. The range of concentrations of ATP was $0.15\text{–}7.25 \times 10^{-3}$ M at 37 and 75°. The range of concentrations of dThd was $0.22\text{–}4.48 \times 10^{-6}$ M at 37° and 32×10^{-6} M–1.58×10^{-3} M at 75°. The initial velocity measurements at each concentration were run in duplicate. The data were plotted according to a linear transformation of the Michaelis-Menten equation (Lineweaver and Burk, 1934). The K_m for Mg-ATP^{2-} was $4.30 \pm 0.21 \times 10^{-4}$ M at 75° and remained constant at $3.77 \pm 0.37 \times 10^{-4}$ M at 37°. The reaction mixtures (60 μl) contained 5 μmol of Tris-Cl (pH 7.6), 50 nmol of [^{14}C]deoxythymidine, varying amounts of ATP-Mg (ATP/Mg 0.41), from 9 to 435 nmol, and 1 μg at 37° or 0.33 μg at 75° of enzyme (fraction V after dialysis). The reactions were incubated at 37° for 90 min, and at 75° for 5 min. The K_m for dThd was $4.79 \pm 0.82 \times 10^{-5}$ M at 75° and $5.26 \pm 0.56 \times 10^{-7}$ M at 37°. For the determination of K_m at 37°, the reaction mixtures (60 μl) contained 5 μmol of Tris-Cl (pH 7.6), 420 nmol of $MgCl_2$, 172 nmol of ATP, varying amounts of deoxythymidine (from 13.2 to 268 pmol), and 30 ng of enzyme (fraction V after analysis). The reactions were incubated at 37° for 5 min. For the determination of K_m at 75°, the reaction mixture (60 μl) contained 5 μmol of Tris-Cl, 420 nmol of

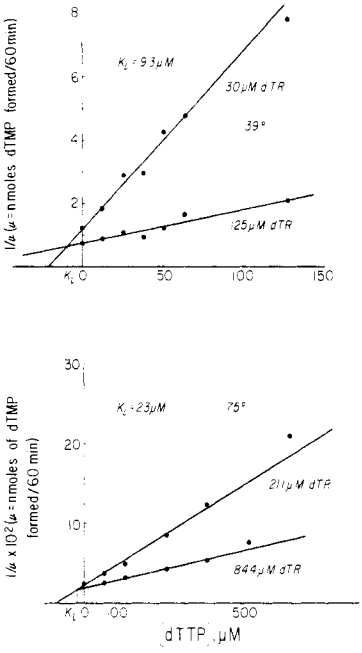

FIGURE 3: Graphical determination of dTTP inhibition constant at 39 and 74.5°. The reaction mixtures (60 μl) contained 5 μmol of Hepes-NaOH (pH 7.58), 420 nmol of $MgCl_2$, 172 nmol of ATP, varying amounts of dTTP (from 0.76 to 7.6 nmol), 1.81 nmol of [^{14}C]deoxythymidine, and 0.06 μg of enzyme (fraction VII). The reactions were incubated at 39° for intervals of time ranging from 15 to 90 min, in order to maintain the extent of conversion to product comparable and below 5%. The experiment was repeated as described, but the amount of [^{14}C]deoxythymidine was increased to 7.62 nmol. The conditions of the experiment carried out at 74.5° were identical with those of 39° except the amounts of dThd used were 12.7 and 50.6 nmol of [^{14}C]thymidine and amounts of dTTP varied from 3.8 to 38 nmol. The time of incubation was 2 and 5 min, respectively.

$MgCl_2$, 172 nmol of ATP, and varying amounts of deoxythymidine (from 1.92 to 95.1 nmol), and 0.33 μg of enzyme (fraction V after dialysis). The reactions were incubated at 75° for 10 min. A 100-fold decrease of the K_m of dThd was noted at 37° in comparison to the value obtained at 75°.

dTTP has been shown to be an affective inhibitor of the kinase purified from mammalian cells (Ives *et al.*, 1963; Breitman, 1963; Bresnick and Thompson, 1965) and *Escherichia coli* B (Okazaki and Kornberg, 1964b). The *bacillus* deoxythymidine kinase is also inhibited by dTTP. Since *B. stearothermophilus* is an obligate thermophile and does not grow at 37°, it was of interest to study the effect of temperature on the efficiency of the kinase activity regulation. Graphic determinations of the inhibition constants at 37 and 75° (Dixon, 1953) are given in Figure 3. The inhibition appears to be competitive with dThd and less efficient at higher temperatures.

Thermostability and Thermophilicity of Deoxythymidine Kinase. The thermostability of the catalytically active conformation of the kinase is remarkable. No loss of activity was detected after an exposure of 60 min at 75°. Even at 100° for 30 min the enzyme lost only 40% of its activity. Curves of the thermostability at various temperatures are given in Figure 4.

The enzyme also exhibits a remarkable degree of thermophilicity. When the catalytic power of the protein was measured at temperatures varying from 32 to 90°, the maximal velocity increased from 1.25 to 220 μmol of dTMP formed hr^{-1} μg^{-1}. The results are given in Figure 5.

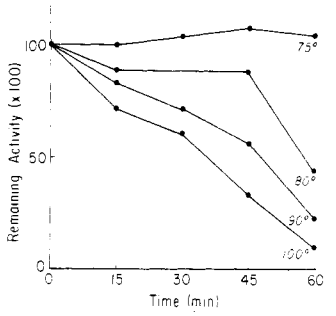

FIGURE 4: Thermostability of deoxythymidine kinase. Fraction VII was diluted 1:6 with 0.1 M Hepes-NaOH buffer (pH 7.6). Portions of 0.6 ml were exposed to 100, 90, 80, and 75° in a plastic centrifuge tube which was tightly stoppered with a screw cap. Fractions of 100 μl were taken at 15, 30, 45, and 60 min. The enzymatic activities were measured by the optical assay.

Discussion

The reported purification produces a deoxythymidine kinase that is adequate for chemical studies. The enzyme appears to be composed of a multiple of polypeptide chains, each weighing 28,500. Whether the polypeptide chains are identical is currently under investigation. Protein concentration and perhaps pH regulate the state of aggregation of the enzyme. In dilute solution at pH 8.3 and 4°, the active form of the enzyme is a dimer of 52,000 as measured by molecular sieve chromatography on Sephadex G-200. The molecular weight, estimated by polyacrylamide gel electrophoresis, indicated that the enzyme can exist as a tetramer. Two size isomers of 54,000 and 108,000 were detected at pH 10.2. The molecular weight of the dimer is in good agreement with the value of 55,000 ± 5000 found for the calf thymus deoxythymidine kinase (Her and Momparler, 1971). However, much larger values (about 700,000) have been obtained for the kinase derived from animal tumors (Bresnick and Thompson, 1965) and regenerating rat liver (Bresnick, 1970). It is difficult to ascertain whether these

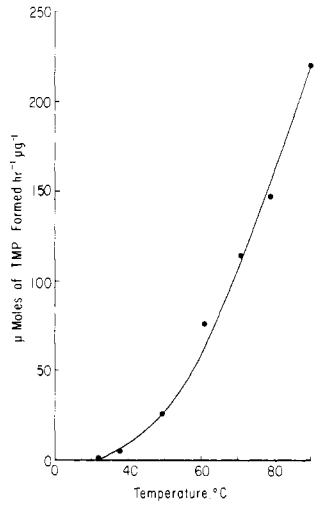

FIGURE 5: Thermophilicity of deoxythymidine kinase. The reaction mixture (60 μl) contained 5 μmol of Hepes-NaOH buffer (pH 7.58), 420 nmol of $MgCl_2$, 50 nmol (cpm 33,643) of [^{14}C]deoxythymidine, and 0.0606 μg of enzyme. Before incubation the reaction was started by the addition of 180 nmol of ATP, in a volume of 10 μl. The mixtures were incubated at 32, 38, 49.5, 61, 71, 79, and 90°. The time of incubation varied from 60 min at 32° to 2 min at 90°; 1 or 2% conversion to product was allowed in order to measure initial velocities. To monitor and to correct for evaporation, the total counts were determined at the end of the incubations at 32, 71, and 90°.

large aggregates are artifacts of the purification procedure or physiologically active complexes.

The affinity of the thermophilic kinase for substrates and regulatory ligands closely resembles its counterpart derived from mammalian cells. The nucleoside triphosphate dTTP is a very effective inhibitor of the calf thymus enzyme, having a K_i of 6.0×10^{-6} M (Her and Momparler, 1971). The thermostable deoxythymidine kinase has a K_i of 9.3×10^{-6} M at 39° and 23×10^{-6} M at 75°. The apparent K_m value of dThd measured at the growing temperature of the *bacillus* (75°) is $4.79 \pm 0.82 \times 10^{-5}$ M. This value is very close to the 5.7×10^{-5} M (37°) reported for the calf thymus deoxythymidine kinase (Her and Momparler, 1971). However, when the K_m of the *bacillus* enzyme for dThd was measured at 37°, a value of $5.26 \pm 0.56 \times 10^{-7}$ M was found. This value is even lower than the value of $2.7\text{–}3.7 \times 10^{-6}$ M reported for the partially purified kinase from animal tumors (Bresnick and Thompson, 1965). This large (100-fold) temperature-dependent decrease of K_m has been interpreted as an increased affinity of the *bacillus* kinase for dThd since it can not be accounted by the decrease of K_2 at lower temperatures (Figure 5).

It is conceivable that this enhanced affinity of the kinase at temperatures of no growth may play a role in DNA repair (Howard-Flanders, 1968; Witkin, 1969). The genome of *Bacillus stearothermophilus* may be still subject to the damage of ultraviolet light at temperatures of no growth (Beukers *et al.*, 1960; Wacker *et al.*, 1961; Setlow *et al.*, 1965) and the enhanced affinity of kinase may, therefore, increase the efficiency of the DNA repair mechanism.

The remarkable thermostability of the *bacillus* kinase (Figure 5) is of interest. Since a homogeneous mammalian kinase preparation has not been reported, whether thermostability is a property unique to the *bacillus* kinase remains to be investigated.

The calf thymus kinase loses about 50% of its activity when exposed to 45° even in presence of 20% glycerol, which is a known protective agent (Her and Momparler, 1971). The *bacillus* kinase in Hepes buffer (pH 7.6) loses 40% of its activity after 30 min of exposure at 100° (Figure 4). The thermolability of the mammalian kinase could be ascribed to other factors, such as cathepsins which could render the active kinase more thermolabile. At the present time, comparison between thermostable proteins and their counterparts isolated from mesophilic microorganisms has not revealed any major difference (for a review of proteins isolated from thermophilic microorganisms, see Singleton and Amelunxen, 1973).

The property of thermostability could be the consequence of a very subtle molecular mechanism. The tertiary structure of a protein is the consequence of its amino acid sequence and certain amino acid sequences preclude certain conformations. The process of folding of a polypeptide chain, however, should be envisioned as a multistep process. The temporal sequence of the folding steps may be of paramount importance in attaining an active conformation. Moreover, every folding step is conditioned by the previous one. It is conceivable that a specific sequence of amino acids may have several folding pathways because of the temporal sequence of its folding steps. As a result of a few amino acid substitutions, thermostable proteins, in contrast to their thermolabile counterparts, may have sequences which would reduce or abolish the ambiguity of certain important folding steps. Because of a unique pathway of folding, the thermostable protein may reversibly fold and unfold without appreciable loss of activity. Disulfide bridges or some strategically located amino acid side chains could preclude some interactions which may yield a tertiary structure catalytically inactive.

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The Phosphorylation of Ribavirin by Deoxyadenosine Kinase from Rat Liver. Differentiation between Adenosine and Deoxyadenosine Kinase†

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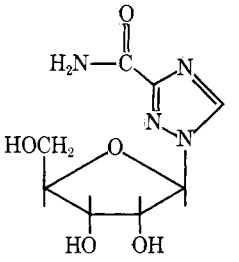
ABSTRACT: A nucleoside kinase activity has been partially purified 30-fold from rat liver which is capable of phosphorylating the synthetic nucleoside, ribavirin. This activity copurifies with both adenosine and deoxyadenosine kinase. The K_m values for the three substrates were: ribavirin, 3.2 mM; deoxyadenosine, 0.50 mM; and adenosine, 0.76 μM. The pH and $MgCl_2$ concentration for optimal enzyme activity were the same with either ribavirin or deoxyadenosine as substrates, but were much lower for adenosine phosphorylation. Both ribavirin and deoxyadenosine kinase exhibited the same sensitivity to inactivation by heat or *p*-chloromercuribenzoic acid, and to protection against heat inactivation by dithiothreitol. The adenosine kinase activity was less sensitive to inactivation by *p*-chloromercuribenzoic acid or heat and to protection by dithiothreitol. Kinetic studies revealed that ribavirin and deoxyadenosine competitively inhibited the phosphorylation of one another, while adenosine was a noncompetitive inhibitor of these two kinase activities. The results indicate that adenosine and deoxyadenosine kinase are separate enzyme activities and that the phosphorylation of ribavirin is associated with the latter activity.

The synthesis and antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (I, ribavirin)[1,2] have been



I, ribavirin

reported (Witkowski *et al.,* 1972; Sidwell *et al.,* 1972) demonstrating that this synthetic nucleoside is a potent inhibitor of a wide variety of RNA and DNA viruses. Studies on the mechanism of antiviral action of this drug in mammalian cell culture (Streeter *et al.,* 1973a) revealed that the 5′-phosphate of ribavirin was a potent inhibitor of the enzyme IMP dehydrogenase, and that the antiviral activity of ribavirin might be due to the inhibition of GMP biosynthesis in virus-infected cells. This would, in turn, result in the inhibition of virus nucleic acid synthesis. Some preliminary evidence that ribavirin could indeed be phosphorylated *in vivo* was presented (Streeter *et al.,* 1973a). Since the 5′-phosphate of ribavirin appears to be the active antiviral agent, the enzymic phosphorylation of ribavirin became of prime interest. Furthermore, both the mechanism of action studies, as well as X-ray crystallography studies on ribavirin (Prusiner and Sundaralingam, 1973), indicated a structural similarity to guanosine or inosine. It was therefore of interest to determine which naturally occurring nucleoside kinase activity was responsible for the phosphorylation of ribavirin, in order to further determine the structural analogy of ribavirin to naturally occurring nucleosides.

A nucleoside kinase activity has been found in crude extracts from rat liver (Streeter *et al.,* 1973b) which phosphorylates ribavirin, and it is the purpose of this paper to report the partial purification and characterization of this enzyme activity.

Experimental Section

Materials. ^{14}C- and ^{3}H-labeled purine and pyrimidine nucleosides were obtained from ICN Pharmaceuticals, Inc., Life Sciences Group. Ribavirin was synthesized according to the method of Witkowski *et al.* (1972). [^{3}H]Ribavirin was obtained by exchange of the unlabeled material in [^{3}H]H_2O. The exchanged material was separated from unexchanged tritium by repeated evaporation from water. The final product was recrystallized from ethanol. Nonradioactive nucleosides and deoxynucleosides, ATP, phosphoenol pyruvate (trisodium salt), and pyruvate kinase

† From the ICN Pharmaceuticals, Inc. Nucleic Acid Research Institute, Irvine, California 92664. *Received May 28, 1974.*

[1] Referred to in previous publications as ICN 1229 or by the ICN Pharmaceuticals, Inc. trademark: Virazole®.

[2] Abbreviations used are: RV, ribavirin; Ado, adenosine; dAdo, deoxyadenosine; PCMB, *p*-chloromercuribenzoate.