Regulation of a Thermostable Pyrimidine Ribonucleoside Kinase by Cytidine Triphosphate†

Antonio Orengo and G. F. Saunders*

ABSTRACT: Pyrimidine ribonucleoside kinase was purified 300-fold from extracts of the obligate thermophile, *Bacillus stearothermophilus*, and several properties of the enzyme were compared with pyrimidine ribonucleoside kinase of comparable specific activity derived from Novikoff ascites rat tumor. The thermophile enzyme appears to be particularly suitable for studies of mechanisms of regulation of catalytic activity. Although both enzymes catalyze the same reaction and are inhibited by the same regulator, *i.e.*, CTP, they display distinctly different kinetic properties. The bacterial enzyme is stable at 59.5° for at least 30 min, while the mammalian enzyme under identical conditions loses 80% of its activity in 4 min. The kinetics of saturation of the thermophile enzyme by ATP is strongly dependent upon temperature and there appears to be more than one level of saturation. Studies on the kinetics of inhibition by CTP at different temperatures revealed that as the temperature is lowered, the thermophile enzyme becomes progressively more insensitive to inhibition by CTP. Inhibition of the mammalian enzyme by CTP was not dependent upon temperature. A break in the Arrhenius plot indicating two activation energies was observed for both enzymes. Although the enzymes had practically the same activation energies, the break in Arrhenius plot was at 46° for the thermophile enzyme and at 35° for the mammalian enzyme. These properties of the thermophile enzyme indicate that the enzyme may undergo marked conformational changes with changes in temperature.

The terminal product of pyrimidine ribonucleotide biosynthesis, CTP, appears to regulate its own synthesis, acting as a specific inhibitor of the first step of the enzymic sequence. By such a mechanism, both the synthesis *de novo* of pyrimidine nucleotides from aspartate, carbon dioxide, ammonia, and 5-phosphoribosyl pyrophosphate (Gerhart and Pardee, 1962) and the salvage pathway (Orengo, 1966) are controlled by the inhibitor. A pyrimidine ribonucleoside kinase (uridine kinase, EC 2.7.1.48) is the first enzyme of the salvage pathway, and catalyzes the following reaction: UR (CR) + ATP → UMP (CMP) + ADP.

Uridine kinase purified from Novikoff ascites rat tumor has been studied extensively by "kinetic" methods. It appears to have at least three distinct binding sites: one for the phosphate acceptor (uridine or cytidine), one for the phosphate donor (ATP, or other nucleoside triphosphates), and one for the regulatory ligand (CTP or UTP) (Orengo, 1969).

Our efforts have been directed toward determining how CTP regulates the catalytic activity of uridine kinase. Of several mammalian cell lines and bacterial species screened, *Bacillus stearothermophilus* was found to have the highest level of uridine kinase activity. In previous work (Saunders *et al.*, 1969) we showed that the purified pyrimidine nucleoside phosphorylase of *B. stearothermophilus* is more thermostable and has a lower molecular weight (78,000) than the corresponding enzyme of *Escherichia coli* (mol wt 148,000). Since the optimal growth temperature of the organism is 65°, an increased thermostability of its uridine kinase was expected. This allows kinetic and binding studies of substrates and regulators over a wide range of temperatures. Thus a comparison of the enzyme from the thermophile and the tumor might contribute to our understanding of the mechanism of regulation. This paper will report the partial purification of the thermophile enzyme, and a comparison of some of its properties with those exhibited by a preparation of uridine kinase of similar specific activity derived from Novikoff ascites rat tumor.

Experimental Procedure

Materials Uridine-2-^{14}C and cytidine-2-^{14}C were purchased from Schwarz BioResearch, Inc. Streptomycin sulfate was obtained from E. R. Squibb and Sons. Calcium phosphate gel and DEAE-cellulose DE-52 were purchased from Calbiochem and Whatman, respectively. All other nucleosides and nucleotides used were reagent grade products. Lactate dehydrogenase was purchased from P-L Biochemicals, pyruvate kinase from Mann, and sodium phosphoenolpyruvate from Sigma. Novikoff ascites tumor (Birns, 1961) and *B. stearothermophilus* strain 10 were originally supplied by Dr. Alex B. Novikoff and Dr. L. L. Campbell, respectively.

Assay of the Enzyme. Two types of enzymic assay, both previously described (Orengo, 1969), were used. The radiochemical assay measures the conversion of nucleoside to nucleotide by chromatographic separation of the reactants and products on DEAE-cellulose paper. The optical assay measures the oxidation of DPNH. In the latter assay, pyruvate kinase is coupled with lactate dehydrogenase in order to measure ADP. Disappearance of DPNH, as measured by a decrease in absorbance at 340 mμ, is a measure of uridine kinase activity. Protein was determined by the method of Lowry *et al.* (1951).

Sucrose Density Gradient Centrifugation. Linear gradients of sucrose from 5 to 20% made up in 0.1 M Tris-HCl (pH 7.6) were centrifuged in a SW50 rotor in a Beckman Model L2-65 preparative ultracentrifuge at 4°. After termination of the run, the gradients were fractionated by use of an ISCO

† From the Departments of Biochemistry and Developmental Therapeutics, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025. *Received May 24, 1971.* This work has been supported by Research Grant CA-10407 and CA-12429 from the National Institutes of Health, U. S. Public Health Service, Grants G-267 and G-460 from The Robert A. Welch Foundation, and Grant T-465A from the American Cancer Society.

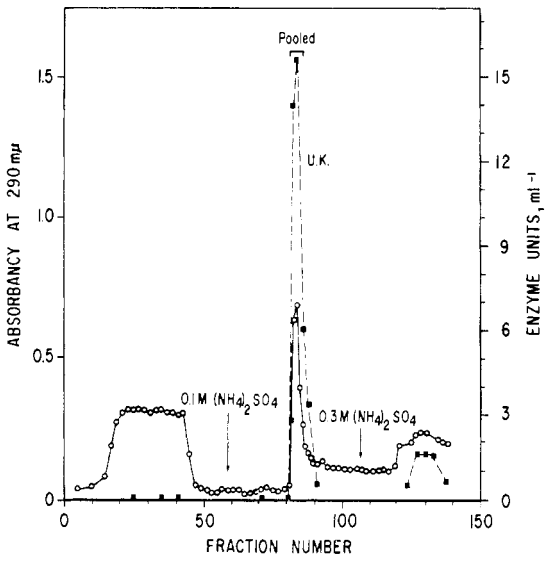

FIGURE 1: DEAE-cellulose chromatography of *B. stearothermophilus* uridine kinase. An aliquot of fraction IV containing approximately 30 mg of protein was applied to a DEAE-cellulose column (4.9 cm^2 × 5.5 cm) equilibrated with 0.1 M Tris-HCl–2 mM uridine–10% glycerol (pH 7.2). After washing with the charging buffer, elution was effected by stepwise addition of ammonium sulfate. The flow rate was 1 ml/min; 2-ml fractions were collected and analyzed as described in the Experimental Procedure section.

TABLE I: Purification of Uridine–Cytidine Kinase from *B. stearothermophilus*.[a]

Fractions and Steps	Act. (Units/ml)	Total Act. (Units)	Protein (mg/ml)	Sp Act. (Units/mg of Protein)
I Extract	1.4	772	7.0	0.2
II Streptomycin	1.3	916	2.46	0.5
III Ammonium sulfate	11.5	402	1.71	6.7
IV Calcium phosphate gel	16.4	205	0.75	21.9
V DEAE-cellulose chromatography	24	184	0.40	60.0

[a] The radiochemical assay as described in the text was used, with uridine as substrate.

density gradient fractionator. The fractions were assayed for enzyme activity as described in the legend of Figure 3.

Growth of Tumor Cells and Preparation of Tumor Enzyme. The Novikoff transplantable ascites cells were grown for 6 days in the peritoneal cavities of young, female, Holtzmann Sprague–Dawley rats (120–150 g in weight.) Preparation of the crude extracts and the purification procedure have already been described (Orengo, 1969).

Growth of Bacterial Cells. B. stearothermophilus strain 10 was grown in a trypticase medium (Saunders and Campbell, 1966) at 60° in a 24-l. laboratory fermentor to an optical density of 0.9–1.2 at 540 mμ. The cells were chilled with ice, harvested by continuous flow centrifugation, washed once with 0.2 M NH_4Cl, and stored at −20°.

Preparation of Bacterial Crude Extracts. Frozen cells were suspended in 0.05 M Tris-HCl (pH 7.5), 2 ml/g, and disrupted by four 15-sec bursts with a Branson sonifier, Model W-185C. Cell debris was removed by centrifugation at 10,000*g* for 15 min. The preparation was adjusted to 0.2% in deoxycholate and then centrifuged at 123,000*g* for 2 hr to prepare the crude enzyme. Protein concentration, usually around 20 mg/ml, was adjusted to 7.0 mg/ml with distilled water (fraction I). The purification procedure of uridine kinase from bacterial extracts is similar but not identical with the one used to prepare the mammalian enzyme, and therefore it will be described here.

Streptomycin Treatment. To 1 l. of fraction I, 250 ml of 5% streptomycin sulfate was added with continuous stirring. The suspensions were stored at least 1 hr and then centrifuged at 20,000*g* for 20 min (fraction II).

Ammonium Sulfate Treatment. Fraction II was brought to 35% saturation by the slow addition with continuous stirring of ammonium sulfate. The suspension was stored for 1 hr and then centrifuged at 20,000*g* for 20 min. The precipitate was dissolved in 0.01 M Tris-HCl (pH 7.6) (Tris buffer). Protein concentration was adjusted to 1.5 mg/ml.

For each 100 ml of fraction II, 10.85 g of ammonium sulfate was added with continuous stirring. The suspension was stored for 30 min and then centrifuged at 20,000*g* for 10 min. To the supernatant, 9.6 g of ammonium sulfate was added and the suspension was allowed to stand for 10 min and centrifuged again at 20,000*g* for 10 min. The pellet was dissolved in Tris buffer (fraction III) and the protein concentration adjusted to 1.0 mg/ml.

Calcium Phosphate Gel Treatment. For each 100 ml of fraction III, 7.5 ml of calcium phosphate gel (32 mg/ml) was added slowly with constant stirring. After 45-min stirring, the suspension was centrifuged for 10 min and the sediment was discarded. To the supernatant, an additional 15 ml of calcium phosphate gel was added. After 45-min stirring, the sediment was collected by centrifugation and dissolved in 0.1 M EDTA–1 × 10^{-3} M uridine (pH 7.4) in a volume equal to fraction III. After brief centrifugation to remove some of the insoluble material, the clear supernatant fraction was brought to 60% saturation with saturated ammonium sulfate solution in 0.01 M Tris-HCl (pH 7.6)–1 × 10^{-3} M uridine. After overnight storage, the sediment was removed by centrifugation and dissolved in a minimal volume of 0.01 M Tris-HCl–10% glycerol (pH 8.0) (fraction IV).

DEAE-Cellulose Chromatography. DEAE-cellulose DE 52 was washed with 0.1 M EDTA (pH 7.0) and then equilibrated with 0.1 M Tris-HCl–10% glycerol (pH 7.24). The resin was washed several times with buffer until the pH and conductance were constant. At the end of the equilibration step, the conductance of the buffer in which the resin was suspended was 4.8 × 10^5 mhos. A column (4.9 cm^2 × 5.5 cm) was prepared and a volume of fraction IV containing about 30 mg of protein was applied to the column at a rate of 1.9 ml/min. The column was then washed with 70 ml of 0.1 M Tris-HCl–10% glycerol–1 mM uridine (pH 7.2). A stepwise gradient of elution was then applied with 0.1 and 1 M ammonium sulfate as limiting concentrations (Figure 1). Volumes of 100 ml were passed through the column for each ammonium sulfate concentration, and 0.1 M Tris-HCl–2 mM uridine–10% glycerol (pH 7.2) was present throughout the elution. The flow rate was 1 ml/min and 2-ml fractions were collected. Since uridine has practically no absorbance at 290 mμ (290/260 = 0.04), the fractions were monitored at 290 mμ to follow the protein elution. Enzymic activity was

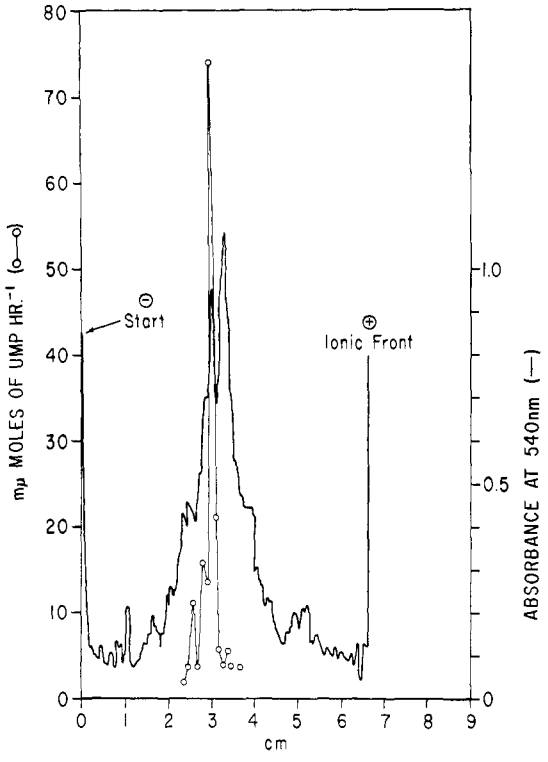

FIGURE 2: Electrophoretic pattern of uridine kinase (fraction V). The electrophoresis was performed by the method of Ornstein (1964) and Davis (1964). The Tris buffer system of Jovin *et al.* (1964) and a 7.5% polyacrylamide sieving gel were used. Protein (50 μg) was applied to the top of the gel together with Bromophenol Blue as tracking dye. At the end of the run the gel was sliced longitudinally. The protein zones in one-half were defined by staining with Coomassie Blue and after destaining the gels were scanned with a Gilford gel scanner. The other half of the gel was frozen and cut into 1-mm thick sections. Each section was allowed to stay at 4° in 0.01 M Tris-HCl (pH 7.6) for at least 1 hr. The enzymic activity of the supernatant fraction was measured as described in the optical assay.

determined by the optical assay. Of the activity applied, 65% was eluted with 0.1 M ammonium sulfate between 4.82 and 5.34 bed volumes of effluent. In preliminary experiments no elution of uridine kinase occurred with 0.6 and 1.0 M, therefore, these steps were omitted in subsequent experiments. Only fractions with the highest specific activity were pooled. The enzyme eluted with 0.1 M was kept apart from the one eluted with 0.3 M ammonium sulfate. All experiments presented in this paper were carried out using pooled fractions of the enzyme eluted with 0.1 M ammonium sulfate (fraction V). The specific activity of fraction V was 300 times that of the cell extract (Table I). The enzyme was stored at 4°. Prior to use aliquots of enzyme were freed of ammonium sulfate and uridine by a chromatography on Sephadex G-25 equilibrated with 0.01 M Tris-HCl (pH 7.6)–10% glycerol. Temperature was maintained at 2–4° throughout the entire isolation procedure.

Results

Purity of Enzyme Preparation. To assess the degree of purification, fraction V was subjected to polyacrylamide gel electrophoresis. The pattern for a 7.5% gel is shown in Figure 2. In separate experiments, under conditions where 95% of the input uridine had been converted to UMP, neither UDP nor UTP could be detected indicating that the enzymic

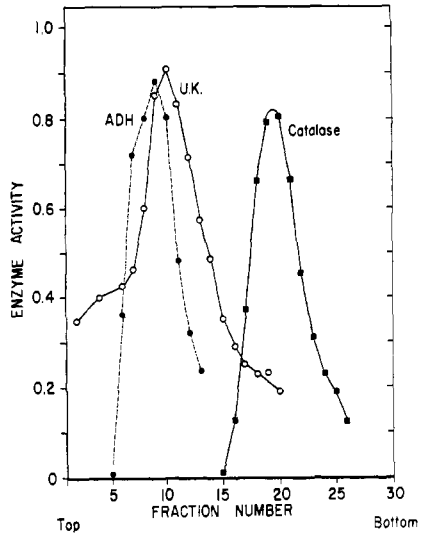

FIGURE 3: Sucrose gradient centrifugation of uridine kinase for approximation of molecular weight. Gradients (5 ml) were layered with 0.15 ml of Tris-HCl (pH 7.6) containing approximately 4700 IU (100 μg) of catalase (Calbiochem), 0.2 unit (100 μg) of liver alcohol dehydrogenase (Worthington), and 0.1 ml of uridine kinase fraction V (60 μg of protein). The gradients were centrifuged for 16 hr at 38,000 rpm at 4°. Fractions of 0.2 ml were collected and assayed for catalase (Beers and Sizer, 1952), alcohol dehydrogenase (Vallee and Hoch, 1955), and uridine kinase (radiochemical assay). Enzyme activity refers to the change in absorbance per minute catalyzed by 0.01 ml of each fraction under the conditions described for the assay of catalase (Beers and Sizer, 1952) and by 1 ml for alcohol dehydrogenase (Vallee and Hoch, 1955).

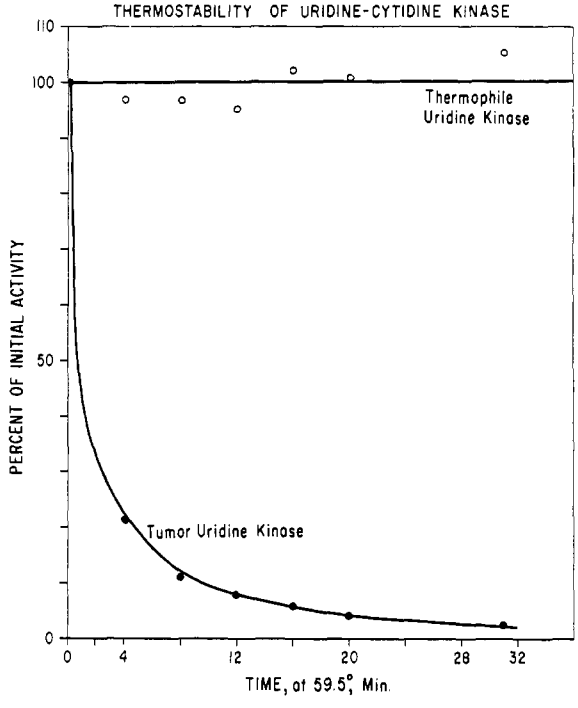

FIGURE 4: Thermostability of uridine–cytidine kinase. Incubation of 100-μl samples of bacterial enzyme, fraction V (0.41 mg/ml), and of tumor enzyme (0.32 mg/ml) in 0.1 M $(NH_4)_2SO_4$–2×10^{-4} M uridine–10% glycerol–0.1 M Tris-HCl (pH 7.2) was performed at 59.5°. At the indicated time intervals, 10-μl aliquots were withdrawn and mixed with 50 μl of ice-cold reaction mixture. After mixing, the reactions were incubated at 37° for 10 min and assayed by the radiochemical method. The reaction mixture (final volume of 50 μl) contained 5 μmoles of Tris-HCl buffer (pH 7.6), 420 nmoles of $MgCl_2$, 150 nmoles of ATP, and 48 nmoles of cytidine-2-^{14}C (1 Ci/mole). The specific activity of the tumor enzyme was 55 μmoles of CMP formed hr^{-1} mg^{-1}.

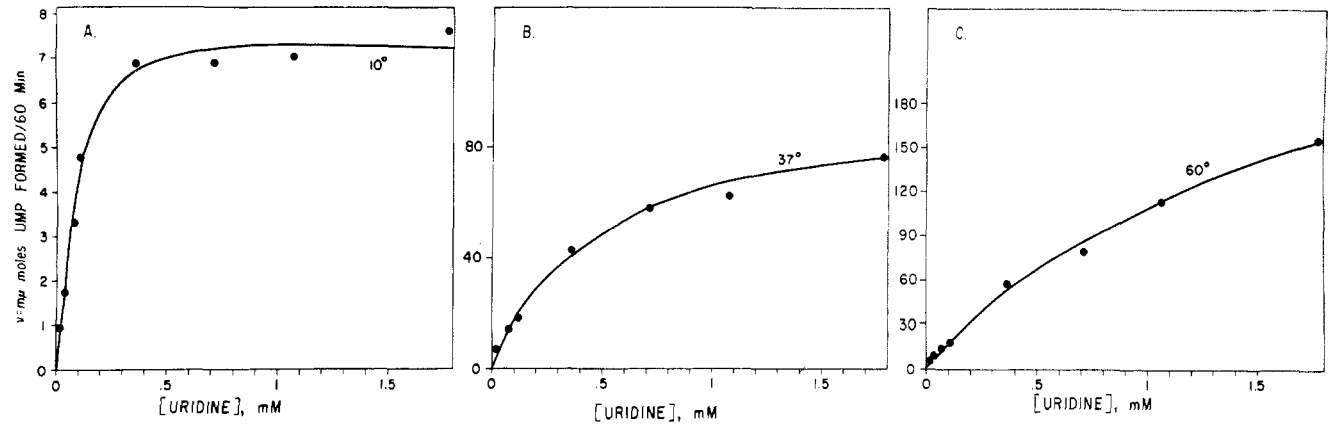

FIGURE 5: Effect of temperature on the uridine saturation curve of thermophile enzyme. The reaction mixture (final volume, 60 μl) contained 5 μmoles of Tris-HCl buffer, 420 nmoles of $MgCl_2$, 600 nmoles of ATP, 4.3 μg of enzyme, and 1.07 to 106.9 nmoles of uridine-2-^{14}C (0.5 Ci/mole). The enzymic preparation was dissolved in 0.01 M Tris-HCl–10% glycerol (pH 7.6). All reactions were carried out at pH 7.6, measured at the reaction temperature. The incubations were carried out for 60, 15, and 10 min at 10°, 37°, and 60°, respectively.

preparation was free of ribonucleotide monphosphokinase. The enzymic preparation did not show any detectable activity as pyrimidine nucleoside phosphorylase or 5′-nucleotidase.

Approximation of Molecular Weight. The method of Martin and Ames (1961) was used to estimate the molecular weight of uridine kinase. The sedimentation profiles are shown in Figure 3. With liver alcohol dehydrogenase as the marker enzyme [mol wt 84,000 (Castellino and Barker, 1968)], the molecular weight of the bacterial enzyme was calculated to be 109,000 at the center of the main peak. The shoulder at the left of the peak indicates that a part of the activity is associated with slower moving molecules. No attempt to furnish an interpretation of this finding has been made in the present context, although it is not inconceivable that at 4°, running temperature of the sucrose gradient, the thermophile enzyme is in more than one molecular weight complex with a predominance of the heavier forms.

Thermostability of Uridine–Cytidine Kinase. Purified preparations of uridine kinase derived from *B. stearothermophilus* were compared with preparations of approximately the same specific activity from the Novikoff ascites rat tumor with regard to their thermostability. Test conditions and results are presented in Figure 4 and its legend. The enzyme derived from the tumor lost 79% of its activity upon exposure of the enzyme solution at 59.5° for 4 min. In contrast, the bacterial enzyme maintained its activity unchanged even after a 30-min exposure at this temperature.

Effect of Temperature on the Concentration Curve and K_m of the Phosphate Acceptor, Uridine. As shown in Figure 5, a much higher concentration of uridine is necessary to saturate the thermophile enzyme at 60° than at either 37° or 10°. The maximum velocities in μmoles of UMP hr^{-1} mg^{-1} were: 2 at 10°, 25 at 37°, and 66 at 60°. The Michaelis constants for uridine calculated from Hofstee plots, were 2.1×10^{-4}, 6.4×10^{-4}, and 16.0×10^{-4} M, respectively. Since the concentration of uridine was not high enough to attain a plateau at 60° the values of V_{max} and K_m, at this temperature may be less accurate. The apparent Michaelis constant of the tumor enzyme for uridine at 37° and at a concentration of ATP approaching saturation was previously shown to be 2.7×10^{-4} M (Orengo, 1969). The effects of lower temperatures on the K_m (uridine) for the tumor enzyme were not investigated.

Analogous results have been reported by Iwatsuki and Okazaki (1967) for deoxythymidine kinase of *E. coli*. An extremely high concentration of deoxythymidine was required to saturate the enzyme at 37°. A Lineweaver–Burk plot showed a sharp break resulting in two linear regions, and two values of K_m were calculated. At 21° and 0°, the enzyme was saturated at a lower concentration of deoxythymidine and K_m values much lower than those at 37° were obtained. The data were explained by assuming the existence of two forms of the enzyme, a form with a low K_m and a less active form with a high K_m. As the temperature is lowered, the values of K_m decrease; this finding was attributed to increased affinity of the enzyme for its substrate. However, it may be pertinent to point out that K_m is not synonymous with K_s, and a change in K_m does not necessarily mean a change in affinity, since in many cases, the dissociation constant of the enzyme–substrate complex into enzyme and product ($K_{catalytic}$) may contribute significantly to the value of K_m.

Effects of Concentration of ATP at Several Temperatures. In contrast to the results with uridine (the phosphate acceptor) the effect of high ATP concentrations (the phosphate donor) were complex at all temperatures and at both low and high concentrations of uridine (Figure 6A and B). At temperatures below the temperature of optimal growth of the thermophilic bacterium, it appears that there is more than one level of saturation for the enzyme. At growth temperature, *i.e.*, 60°, the saturation curve tends to be sigmoidal.

It should be pointed out that at low concentrations of ATP (insets of Figure 6A,B) Michaelis kinetics were exhibited. The experiments reported here were repeated three times with essentially the same results and studies on course of the reaction showed that with one exception true initial rates were being measured. At 60° 1.7 mM uridine is not sufficient to saturate the enzyme (Figure 5C). This accounts for the lower initial velocity observed at 60° in low uridine concentration (Figure 6A, inset) as compared with the initial velocity at 38°. The curves are unusual and some of them cannot be handled satisfactorily with the available mathematical models. The experiment is reported here only to point out the distinct effect of temperature on the ATP saturation curve of the thermophile enzyme. Since these curves are reproducible and represent trends it has been difficult for us to dismiss them as trivial artifacts. Work is in progress to find an explanation in terms of conformational transitions. In

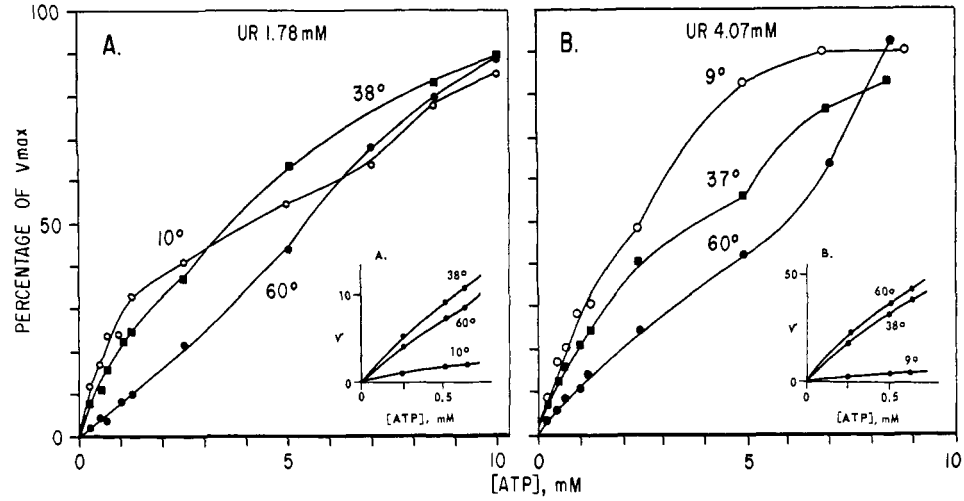

FIGURE 6: Effect of temperature on the ATP saturation curve of the thermophile enzyme. The conditions were described in Figure 5 except 107 nmoles (A) or 244 nmoles (B) of uridine-2-^{14}C (0.5 Ci/mole), 2.1 μg of enzyme, and 15 to 600 nmoles of ATP were used. The incubations were carried out for 60, 15, and 10 min at 11°, 38°, and 60°, respectively.

contrast, the tumor enzyme exhibited Michaelis kinetics and saturation was reached below 3 mM ATP (Figure 7).

CTP Inhibition. Both the thermophile and the tumor UK are inhibited by CTP. Figure 8 shows that low concentrations of CTP are much more inhibitory to the thermophile enzyme than to the tumor enzyme. In contrast, high CTP concentration will almost completely inhibit the tumor enzyme while inhibiting only 80–85% of the thermophile enzyme activity (Figure 8A). A study of inhibition by CTP at different temperatures revealed that as the temperature is lowered, a progressively larger fraction of the thermophile enzyme becomes insensitive to inhibition by CTP (Figure 8C). The tumor enzyme did not show a comparable temperature dependency of the inhibition (Figure 8b).

Effect of Temperature on Catalysis. When initial velocities were measured at a series of temperatures at substrate concentrations close to saturation, the Arrhenius plot presented a break from which two activation energies of uridine kinase could be calculated. The tumor differs from the thermophile enzyme in having the inflection point at a lower temperature. The activation energies are practically the same for both enzymes and are lower at the higher temperatures. The values were 8.5 and 14.2 kcal per mole for the thermophile enzyme and 7.8 and 14.9 kcal per mole for the tumor enzyme (Figure 9).

This phenomenon (*i.e.*, a break in the Arrhenius plot) has been reported previously. Massey *et al.* (1966) listed several enzymes which show a sharp transition between two linear regions of the Arrhenius plot. It may be possible to account for such behavior in terms of changes in the tertiary and/or quaternary structure of the protein (Massey *et al.*, 1966).

Discussion

The thermophile pyrimidine ribonucleoside kinase is an example of environmental influence on macromolecular evolution where cells selected means of stabilizing proteins at high temperature in conformations catalytically active and at the same time amenable to activity regulation.

The finding that some enzymic proteins may form complexes with ligands which are neither substrates nor products of the reaction catalyzed is of interest in that one may exploit molecular mechanisms in chemical homeostasis. The ability of the protein molecule to form such complexes may regulate the activity of a reaction sequence if the ligand is the terminal product and interacts with the first catalyst of the sequence. Alternatively it may allow coordination of different sequences of chemical transformations if the ligand is a product of a sequence and interacts with the catalyst of another sequence.

Enzymes from thermophilic organisms are of particular interest since these proteins must be in conformations which are catalytically active at the elevated temperatures needed for growth. Since several end-product-regulated enzymes prepared from mesophilic bacteria lose regulation but maintain catalysis upon mild heat treatment *in vitro* (Changeux,

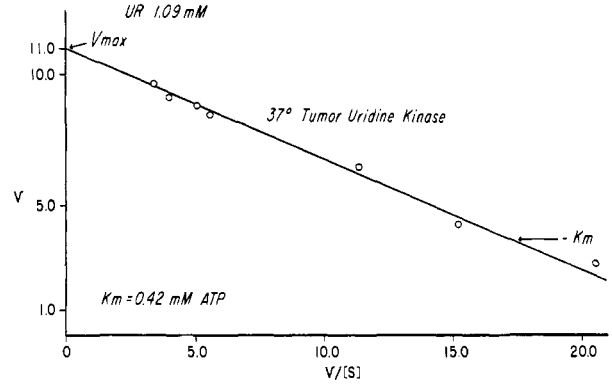

FIGURE 7: Effect of ATP concentration on mammalian enzyme. The optical assay was used. The standard assay mixture (final volume 1.0 ml) contained the following: 100 μmoles of Tris-HCl buffer (pH 7.5), 7.0 μmoles of $MgCl_2$, 15.0 μmoles of KCl, 3.0 μmoles of sodium phosphoenolpyruvate, 7 μg of pyruvate kinase (1930 enzyme units per mg), 10 μg of lactate dehydrogenase (300 units per mg), 0.128 μg of DPNH, 110 μg of enzyme, 109 μmoles of uridine, and 0.142–2.85 μmoles of ATP. The reaction was initiated by adding the nucleoside and the decrease in absorbance at 340 mμ was monitored. The reaction was measured at 37° and care was taken to use batches of ATP and phosphoenolpyruvate containing only minimal amounts of ADP or pyruvate. Blank runs without uridine were stable at 37°, showing no decrease in absorbance at 340 mμ over a period of at least 30 min.

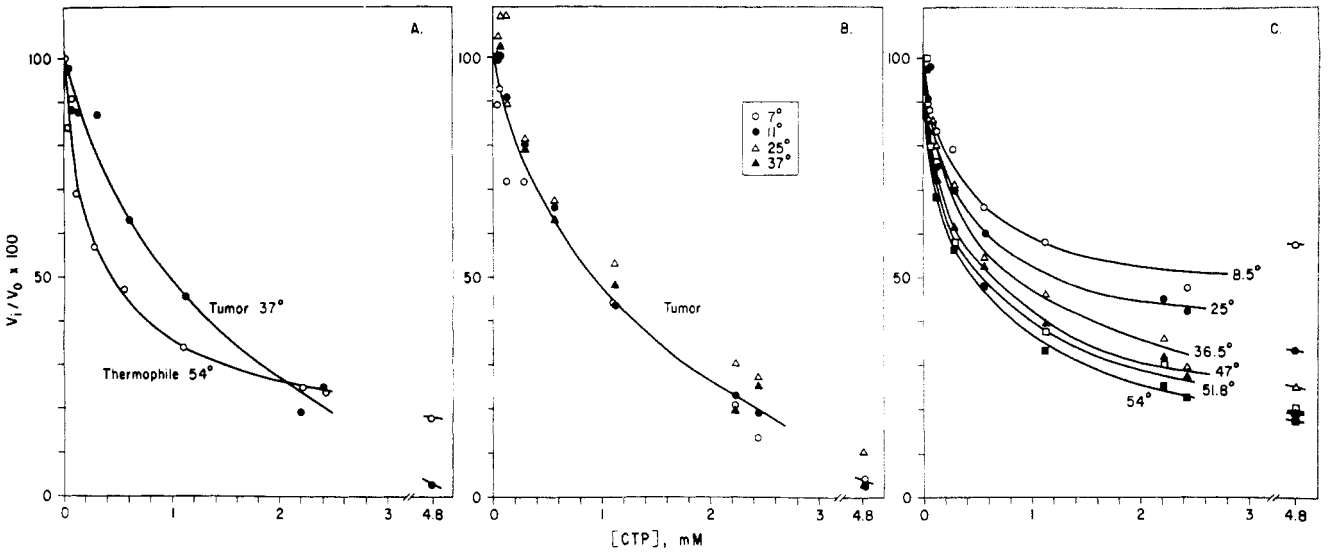

FIGURE 8: Effect of CTP concentration on uridine kinase activity. The reaction mixture (final volume, 60 μl) contained 5 μmoles of Tris-HCl buffer, 42 nmoles of cytidine-2-^{14}C (1 μCi/mole), 150 nmoles of ATP, 1.7–292.6 nmoles of CTP, and 2 μg of the thermophile enzyme or 0.6 μg of the tumor enzyme. All reactions were carried out at pH 7.6 and assayed by the radiochemical method. Panel A compares the tumor and thermophile enzyme at temperatures of growth. Panel B compares the tumor enzyme at several temperatures. Panel C compares the thermophile enzyme at several temperatures.

1961; Patte *et al.*, 1963), it appears pertinent to investigate the regulation of enzymes catalyzing the same reaction but derived from thermophilic microorganisms. These comparisons can lead to the understanding of both the mechanism of action of enzymes and their regulation of activity. The findings presented in this paper represent initial steps toward these goals.

The studies on kinetics reported here point out that the mesophilic and thermophilic pyrimidine ribonucleoside kinases display distinct kinetic differences which have implications with regard to both the regulatory and catalytic site, although both enzymes carry out the same reaction.

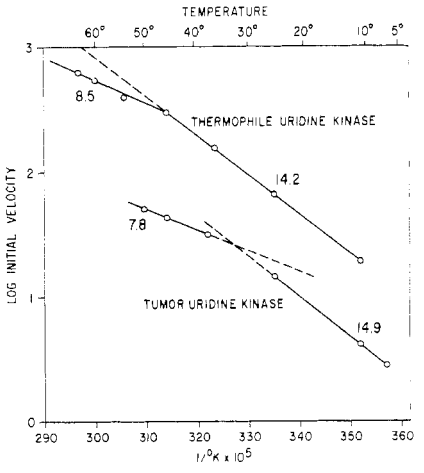

FIGURE 9: Effect of temperature on catalysis. The thermophile enzyme reaction mixtures (final volume, 60 μl) contained 5 μmoles of Tris-HCl buffer, 840 nmoles of $MgCl_2$, 600 nmoles of ATP, 326 nmoles of uridine-2-^{14}C (0.5 Ci/mole), and 6.7 μg of fraction V. The reaction mixtures for the activity assay of the tumor enzyme (final volume, 60 μl) contained 5 μmoles of Tris-HCl buffer, 420 nmoles of $MgCl_2$, 150 nmoles of ATP, 48 nmoles of cytidine-2-^{14}C (1 μCi/mole), and 0.6 μg of enzyme. The enzyme solutions were in 0.1 M $(NH_4)_2SO_4$–0.002 M uridine–10% glycerol–0.1 M Tris-HCl (pH 7.2). All reactions were carried out at pH 7.6. The reaction mixtures were incubated at different temperatures and time intervals and assayed by the radiochemical method.

Below the growth temperature (43°) of the obligate thermophile the end product regulation became much less effective. Concentrations of CTP (4.8 mM) which completely inhibit the mammalian kinase are able to produce only 40% inhibition of the thermophile enzyme at a temperature of 8° The mammalian uridine kinase does not show such desensitization below the physiological temperature of 37°. The thermophilic kinase however, is effectively inhibited at growth temperature. The thermophile kinase again exhibits distinct differences in the kinetic properties of the phosphodonor site. Concentrations of 9–10 mM ATP are required to approach saturation in contrast to 2.8 mM required by the mammalian enzyme. Moreover, there are strong temperature effects on the kinetics of saturation of the thermophile kinase by ATP. At the optimum temperature for bacterial growth the kinase appears to have a sigmoidal saturation curve; below this temperature the kinetic behavior is quite unusual suggesting that two successive levels of saturation are reached.

Two conceivable mechanisms through which such differences could be explained are (a) evolutionary changes in the primary structure, *i.e.*, amino acid substitutions; and (b) changes in the quaternary structure, *i.e.*, shift in association–dissociation equilibria of monomers generated by the presence of stabilizing molecules. A detailed study of the molecular structure of the two kinases will be necessary to permit a choice between these alternatives.

Acknowledgments

We are indebted to Professor Robert B. Hurlbert for helpful criticism of the manuscript and to Miss Frances J. Estes and Mrs. Elsie Wu for excellent technical assistance.

References

Beers, R. F., Jr., and Sizer, I. W. (1952), *J. Biol. Chem. 195*, 133.

Birns, M. (1961), *Exp. Cell Res. 22*, 317.
Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.
Changeux, J.-P. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 313.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Iwatsuki, N., and Okazaki, R. (1967), *J. Mol. Biol. 29*, 155.
Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martin, R. G. (1963), *J. Biol. Chem. 238*, 257.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Massey, V., Curti, B., and Ganther, H. (1966), *J. Biol. Chem. 241*, 2347.
Orengo, A. (1966), *Exp. Cell Res. 41*, 338.
Orengo, A. (1969), *J. Biol. Chem. 244*, 2204.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Patte, J., LeBras, G., Loving, T., and Cohen, G. N. (1963), *Biochim. Biophys. Acta 67*, 16.
Saunders, G. F., and Campbell, L. L. (1966), *J. Bacteriol. 91*, 332.
Saunders, P. P., Wilson, B. A., and Saunders, G. F. (1969), *J. Biol. Chem. 244*, 3691.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 327.

Purification and Properties of Transaldolase from Bovine Mammary Gland†

Ekkehard Kuhn and Karl Brand*

ABSTRACT: Transaldolase (EC 2.2.1.2) was purified from bovine mammary gland to a specific activity of 4 IU/mg by a combination of ammonium sulfate precipitation and chromatography on DEAE-Sephadex A-50, Sephadex G-100, and DEAE-cellulose. This purification procedure was found to be reproducible within ±10%. The enzyme appears to be homogeneous as judged from sedimentation equilibrium and sedimentation velocity experiments in the ultracentrifuge. An aqueous and neutral solution of this transaldolase preparation is stable at 2° for at least 8 weeks. No evidence was obtained for the requirement of a cofactor. The molecular weight (M) determined by equilibrium sedimentation and thin-layer gel chromatography was found to be 65,000 ± 2%. From sedimentation velocity experiments a sedimentation coefficient $s_{20,w}$ of 3.67 S was obtained. The diffusion constant and the frictional ratio were calculated from M and $s_{20,w}$ to be 5.5×10^{-7} cm^2 sec^{-1} and 1.37 S, respectively. Apparent K_m values for the substrates fructose 6-phosphate and erythrose 4-phosphate were determined to be 2×10^{-4} and 7×10^{-6} M. The enzyme is stable between pH 4 and 9 and has an optimum activity at pH 8.2. In contrast to transaldolase from *Candida utilis* transaldolase from bovine mammary gland was completely inactivated by treatment with sodium borohydride at pH 6.0 and 2° in the absence of substrate fructose 6-phosphate. The rate of inactivation, however, was strongly enhanced by the addition of substrate. No radioactively labeled amino acid derivative could be detected in the acid hydrolysate with two different methods, when the enzyme was treated with borohydride in the presence of excess U-^{14}C-labeled fructose 6-phosphate. The results are discussed and compared to the structural and catalytic properties reported from the yeast enzyme.

Transaldolase (D-sedoheptulose 7-phosphate:D-glyceraldehyde 3-phosphate dihydroxyacetone transferase, EC 2.2.1.2) catalyzes fructose-6-P + erythrose-4-P ⇌ sedoheptulose-7-P + glyceraldehyde-3-P.

When transaldolase isolated from *Candida utilis* is incubated with fructose 6-phosphate in the absence of the acceptor, erythrose-4-P, a stable enzymatically active intermediate accumulates (Horecker *et al.*, 1961; Venkataraman and Racker, 1961). This intermediate has been identified as a Schiff base containing dihydroxyacetone linked to the ε-amino group of a lysine residue of the enzyme (Horecker *et al*, 1963). Further evidence for the azomethine structure of the intermediate was presented by demonstrating the addition of HCN to the isolated transaldolase–dihydroxyacetone complex (Brand and Horecker, 1968). Histidine residues in transaldolase from yeast were reported to have a specific function in removing the proton from the C-4 hydroxyl group of fructose 6-phosphate and thus promoting the aldol-cleavage reaction (Brand *et al.*, 1969).

It is known that transaldolase is widely distributed in microorganisms, plants, and animal tissues. So far, however, the enzyme was purified only from various types of yeast cells (Pontremoli *et al.*, 1961, Venkataraman and Racker, 1961; Tsolas and Horecker, 1970). No isolation from animal tissues is reported in the literature.

In the present work a purification procedure of transaldolase from bovine mammary gland is described. Furthermore results obtained from studies on the molecular and kinetic properties of the enzyme and its modification by sodium borohydride are presented.

† From the Max-Planck-Institut für Ernährungsphysiologie, 46 Dortmund, Rheinlanddamm 201 Germany. *Received November 5, 1971.* Dedicated to Professor A. E. Braunstein, Moscow, on the occasion of his 70th anniversary.

* To whom correspondence should be sent.