Biochimica et Biophysica Acta, 350 (1974) 193–200

BBA 67212

PURIFICATION AND PROPERTIES OF THYMIDINE KINASE FROM REGENERATING RAT LIVER

DONALD E. KIZER and LEROY HOLMAN

Biomedical Division, The Samuel Roberts Noble Foundation, Inc. Route 1, Ardmore, Okla. 73401 (U.S.A.)

(Received November 11th, 1973)

(Revised manuscript received February 7th, 1974)

SUMMARY

Thymidine kinase (EC 2.7.1.75) from 36-h regenerating rat liver was purified 2600-fold relative to the specific activity of enzyme in the cytoplasmic fraction. About 8 stained bands were visible after electrophoresis on sodium dodecylsulfate-containing polyacrylamide gels. The molecular weight estimated by centrifugation through linear sucrose gradients was 69 000. K_m estimates for thymidine and ATP were 3.2 μM and 0.3 mM, respectively. The enzyme was inhibited by dTTP; inhibition was non-competitive with respect to the phosphate donor and complex with respect to the phosphate acceptor.

INTRODUCTION

Thymidine kinase (EC 2.7.1.75) has been purified from *Escherichia coli* [1, 2], regenerating rat liver [3], calf thymus [4] and several neoplastic tissues [5, 6]. Among these, only the enzyme from *E. coli* was purified to electrophoretic homogeneity [2]. The kinase from regenerating rat liver was purified about 150-fold relative to its specific activity in the cytoplasmic fraction [3]. This enzyme [3] and the enzyme from *E. coli* [1] showed complex kinetics with respect to the substrate ATP, and both enzymes were inhibited by dTTP [7, 8].

We sought ways to improve the purification of thymidine kinase from regenerating liver. Then we studied some of the kinetic and physical properties of this preparation.

MATERIALS AND METHODS

Partial hepatectomy

Female Holtzman rats weighing 150–200 g were housed in a room with 12-h light and dark cycles, and were fed Teklad Rat and Mouse Diet ad libitum. They were partially hepatectomized [9] 2 h after entering a dark cycle; 36 h later, they were killed by cervical fracture.

Preliminary purification of thymidine kinase

Livers from 25 rats were homogenized in 50 mM Tris buffer, pH 8.0, containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 6 mM mercaptoethanol. The homogenates were centrifuged 30 min at 105 000 $\times$ g; the supernatant was Step I enzyme. This preparation was subjected to 4 purification steps that were similar to the procedure published by Bresnick et al. [3], viz. precipitation with $(NH_4)_2SO_4$ at 30% saturation; heating at 60 °C for 4 min; negative absorption on $Ca_3(PO_4)_2$ gel; and re-precipitation with $(NH_4)_2SO_4$ at 30% saturation. We called this preparation Step V enzyme.

Thymidine kinase assay

Reaction mixtures were formulated essentially in the manner described by Maley et al. [10]. Unless otherwise specified, thymidine, ATP and $MgCl_2$ were present at 400 μM, 20 mM and 20 mM, respectively. The labeled substrate [*Me*-^{3}H]thymidine was present at 10 Ci/mole.

Enzyme purified through Steps V–VIII lost catalytic activity upon dilution. Such losses were minimized by concentrating dilute solutions to 2–4 mg of protein per ml prior to assay or storage, by maintaining mercaptoethanol at 6 mM, and by adding crystalline bovine serum albumin to reaction mixtures at 2 mg/ml. The amount of enzyme added to reaction mixtures was adjusted to assure linear velocity vs time plots through 30 min incubation. Usually incubation was for 15 min at 37 °C. Reactions were stopped by dispersing 0.05-ml aliquots of reaction mixtures on 2 cm $\times$ 2.2 cm rectangles of DEAE-cellulose and dropping them into 20 ml of 1 mM ammonium formate [11]. Papers were washed [11] and counted in a xylene–dioxane–cellosolve phosphor solution [12]. Radioactivity was estimated in a Mark I, Nuclear Chicago instrument; quenching was corrected by channels ratio [13]. The amount of protein was estimated by the procedure of Lowry et al. [14]. When estimating protein content of dilute enzyme solutions, interference from Tris and mercaptoethanol was minimized by pre-treatment of enzyme solutions with 100 mM iodoacetate (0.1 ml enzyme solution, 0.4 ml of water and 0.5 ml of iodoacetate).

Disc gel electrophoresis

The procedure was essentially that described by Albrecht and Van Zyl [15]. After treatment with 8 M urea in 1% sodium dodecylsulfate and 1% mercaptoethanol, about 50 μg of enzyme preparation in 10% sucrose was placed on 10% polyacrylamide gels containing 0.1% sodium dodecylsulfate. Electrophoresis in phosphate–sodium dodecylsulfate buffer [16] was at 5 mA/gel for 4 h at room temperature. Gels were stained with Coomassie blue and were destained in methanol–acetic acid [15].

Molecular weight estimation

Thymidine kinase in 50 mM Tris buffer, pH 8.0, containing 6 mM mercaptoethanol and 2 mM ATP was layered upon linear 0–10% sucrose gradients containing buffer–mercaptoethanol–ATP. The enzyme was layered upon the gradient after centrifugation was begun by using band-forming caps (Beckman Instruments, Inc., Palo Alto, Calif.). Centrifugation was for 11 h at 40 000 rev./min in a Beckman Model L centrifuge using an SW-50L rotor. Molecular weight of thymidine kinase was estimated relative to the migration of albumin using the procedure of Martin and Ames [17].

RESULTS

Purification procedure

The procedure used to purify thymidine kinase from regenerating rat liver is outlined in Table I and is described below. To provide sufficient enzyme for studies described, 15 Step V procedures were necessary; for each procedure, 25 rats were partially hepatectomized. Three experiments were performed in which Step V enzyme was purified further by application of Steps VI–VIII. For each experiment, enzyme from 5 Step-V procedures was pooled. In our hands, Step I–V increased the specific activity of thymidine kinase 237 ± 15-fold, while Steps VI, VII and VIII increased specific activity 2680 ± 280-fold.

TABLE I

PROCEDURES AND EXTENT OF PURIFICATION

Step	Procedure	Total protein	Total units	Spec. act.	Fold purification	Recovery (%)
I	105 000 × *g* supernatant	5300 ± 110*	41 010 ± 1620	8 ± 0.3	—	—
II	$(NH_4)_2SO_4$ precipitation	910 ± 30	37 340 ± 1240	42 ± 1	6 ± 0.1	93 ± 2
III	Heat at 60 °C	230 ± 10	23 860 ± 930	110 ± 4	15 ± 0.6	60 ± 2
IV	Negative gel absorption	40 ± 4	18 156 ± 970	640 ± 50	83 ± 7	45 ± 2
V	$(NH_4)_2SO_4$ precipitation	12 ± 1	18 240 ± 760	1780 ± 100	237 ± 15	46 ±2
VI	Positive gel absorption	5 ± 1**	20 040 ± 4420	7620 ± 1810	980 ± 130	19 ± 2
VII	DEAE-Sephadex chromatography	2.4 ± 0.6	17 020 ± 1940	11 700 ± 1680	1540 ± 150	12 ± 2
VIII	$Ca_3(PO_4)_2$ gel column	0.7 ± 0.2	15 750 ± 4390	20 190 ± 2030	2680 ± 280	5 ± 1

* Values shown through Step V are averages ±S.E. for 15 experiments. For each experiment, 25 rats were partially hepatectomized; they were killed 36 h later and the livers were pooled.

** Values shown through Step VIII are averages ±S.E. for 3 experiments. For each experiment, Step V enzyme from 5 experiments described in * was pooled.

Step VI. $Ca_3(PO_4)_2$ was added to the enzyme solution from Step V (4 mg gel per mg protein) and the mixture was stirred 15 min at 4 °C. In the presence of ATP, thymidine kinase binds to $Ca_3(PO_4)_2$ gel, therefore, the gel was collected by centrifugation at 600 × *g* for 5 min, and was resuspended in 50 mM Tris buffer, pH 8.0, containing 2 mM ATP and 6 mM mercaptoethanol. After stirring briefly, the gel again was centrifuged, and the pellet was resuspended in Tris–ATP–mercaptoethanol buffer containing 1 M NaCl. The mixture was stirred for 15 min at 4 °C and then centrifuged at 12 000 × *g* for 15 min. The $Ca_3(PO_4)_2$ gel pellet was discarded. The supernatant was dialyzed 4 h against 50 mM Tris buffer, pH 10.0, containing 2 mM ATP and 6 mM mercaptoethanol.

Step VII. Enzyme was added to a 2.5 cm × 20 cm DEAE-Sephadex column that had been equilibrated with pH 10.0 Tris–ATP–mercaptoethanol buffer. Thymidine kinase was eluted with a 200-ml linear NaCl gradient (0–0.5 M) containing pH

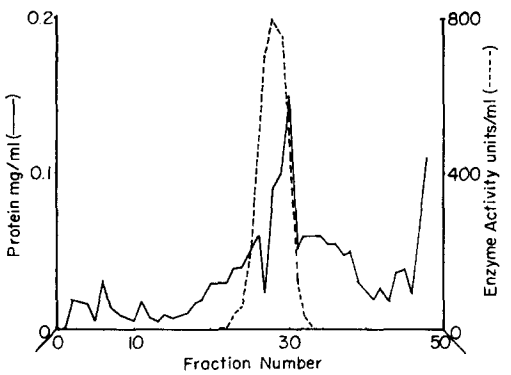

Fig. 1. DEAE-Sephadex chromatography of Step VI thymidine kinase activity. About 5 mg of Step VI enzyme was applied to a 2.5 cm × 20 cm DEAE-Sephadex column that had been equilibrated with 50 mM Tris buffer, pH 10.0, containing 2 mM ATP and 6 mM mercaptoethanol. Enzyme was eluted by passing 200 ml of a linear NaCl gradient (0–0.5 M) developed in pH 10.0 Tris–ATP–mercaptoethanol buffer.

10.0 Tris–ATP–mercaptoethanol buffer. Elution profiles for protein and for enzyme activity are shown in Fig. 1. Enzyme was precipitated from peak fractions by addition of $(NH_4)_2SO_4$ to 30% saturation. Precipitates were collected by centrifugation (12 000 × *g*, 15 min) and were dissolved in 50 mM Tris, pH 8.0, containing 2 mM ATP and 6 mM mercaptoethanol.

Step VIII. $Ca_3(PO_4)_2$ gel was added to the enzyme solution at 4 mg gel per mg protein. The mixture was stirred 15 min at 4 °C and then poured onto a glass filter (2 cm diameter). Enzyme was eluted from the $Ca_3(PO_4)_2$ gel by passage of 40 ml of a linear NaCl gradient (0–1.5 M). Elution profiles for protein and enzyme activity are shown in Fig. 2. Enzyme in peak fractions was concentrated in a membrane ultrafiltration apparatus and dialyzed 4 h against pH 8.0 Tris–ATP–mercaptoethanol buffer.

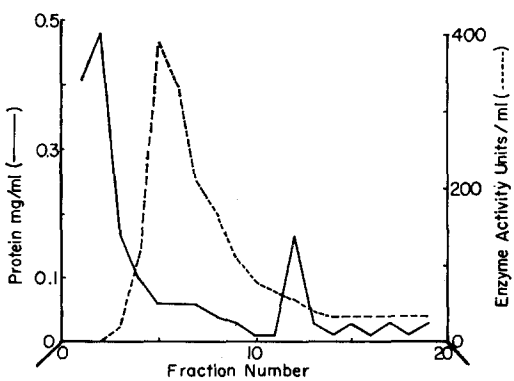

Fig. 2. Elution profiles for protein and thymidine kinase from $Ca_3(PO_4)_2$ gel. About 2 mg of Step VII enzyme in Tris–ATP–mercaptoethanol buffer was stirred with $Ca_3(PO_4)_2$ gel (4 mg gel per mg protein) for 15 min at 4 °C. The mixture was poured onto a glass filter and briefly washed with Tris–ATP–mercaptoethanol buffer. Enzyme was eluted by passing 40 ml of a linear NaCl gradient (0–1.5 M) which was developed in Tris–ATP–mercaptoethanol buffer (pH 8.0).

Disc gel electrophoresis

After electrophoresis on polyacrylamide gels containing sodium dodecylsulfate, 8 stained bands were readily seen. The distribution of these bands on the gel is depicted photographically and diagrammatically in Fig. 3.

Fig. 3. Electrophoresis of Step VIII thymidine kinase on 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate [15]. About 50 μg of protein in 10% sucrose was subjected to electrophoresis for 4 h at 5 mA. A phosphate buffer containing sodium dodecylsulfate [16] was used during electrophoresis. Protein bands were stained with Coomassie blue and destained with methanol–acetic acid [15].

Effect of enzyme concentration and pH

Reaction rates were proportional to the amount of Step VIII enzyme present through a protein concentration of 24 μg/ml in reaction mixtures. The relationship between pH and reaction rate for Step VIII enzyme is illustrated in Fig. 4. Between pH 7.5 and 9.0, reaction rates were about 90% of a maximum value determined at pH 8.0. Below pH 7.5 and above pH 9.0, reaction rates rapidly declined.

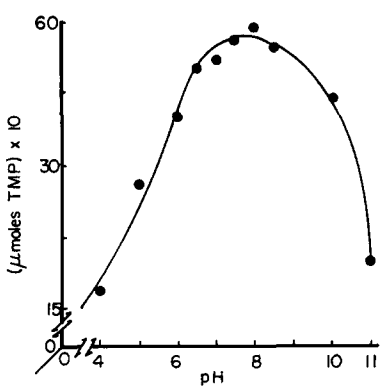

Fig. 4. Influence of pH on reaction rate. Step VIII thymidine kinase was added to reaction mixtures described in Methods. The buffer was 50 mM Tris.

Effect of substrate concentration

The rate of reaction for Step VIII thymidine kinase varied as the concentration of ATP or thymidine was varied. K_m estimates were 3.72 $\pm$ 0.24 μM for thymidine and 0.29 $\pm$ 0.03 mM for ATP.

Step VIII enzyme was inhibited by dTTP. Inhibition was non-competitive with respect to the phosphate donor, Fig. 5A, but was complex with respect to the phosphate acceptor, Fig. 5B. When the concentration of thymidine was decreased from 400 to 20 μM, dTTP inhibition was complex with respect to ATP. In a similar manner,

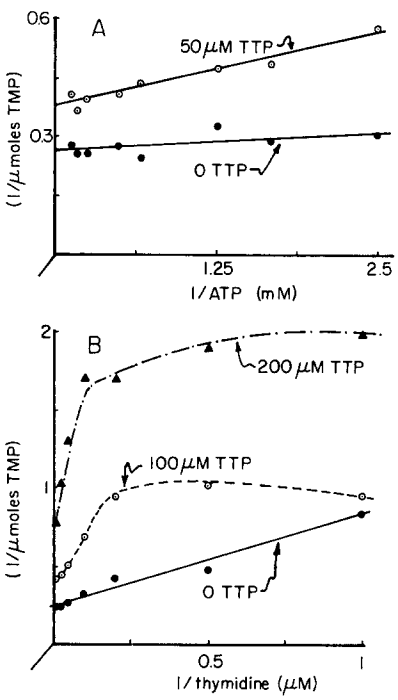

Fig. 5. Inhibition of thymidine kinase by dTTP with respect to the phosphate donor (A), or phosphate acceptor (B). Step VIII enzyme was added to reaction mixtures like those described in Methods except for changes in the ATP concentration in (A) and changes in the thymidine concentration in (B).

when the concentration of ATP was lowered from 20 to 0.5 mM, dTTP inhibition again was complex with respect to thymidine, but the complex nature of dTTP inhibition was intensified. We interpreted these data as indicating substrate cooperativity in the presence of dTTP.

Molecular weight estimation

Molecular weight of Step VIII thymidine kinase was estimated by centrifugation through linear 0–10% sucrose gradients and comparing its mobility to that of bovine serum albumin. Assuming a molecular weight of 67 000 for bovine serum albumin, the molecular weight of thymidine kinase was estimated to be 69 600.

DISCUSSION

Purification procedure

Thymidine kinase purified from *E. coli* was homogeneous by analytical disc gel electrophoresis [2]. Among mammalian tissues, Hashimoto et al. [6] purified the kinase from Yoshida sarcoma about 1500-fold, while the kinase from regenerating liver [3], calf thymus [4] and Ehrlich ascites tumor cells [5] was purified about 150-, 140- and 40-fold, respectively. Purification was improved in the present work, but multiple bands after electrophoresis in sodium dodecylsulfate-containing gels suggested protein heterogeneity. Thymidine kinase isolated from *E. coli* consisted of two subunits which migrated as a single band in sodium dodecylsulfate-containing gels to yield molecular weight estimates of about 46 000 [2]. Bresnick et al. [3] reported a molecular weight of about 80 000 for thymidine kinase from regenerating rat liver and postulated that it too was an oligomer. In view of these considerations, had our purification procedure yielded a homogeneous protein, dissociation of the enzyme with urea and sodium dodecylsulfate followed by electrophoresis should have revealed no more than 2 stained bands; as shown in Fig. 3, 8 stained bands were readily visible.

In the present study, the behavior of the enzyme on $Ca_3(PO_4)_2$ gels afforded considerable advantage in the purification of the enzyme. In the absence of ATP, the enzyme was not bound to the gel and purification was improved about 4-fold over the previous step; in the presence of ATP, the enzyme was bound to $Ca_3(PO_4)_2$ gels, but was eluted with salt. On two occasions that this latter technique was used, viz. Step VI and VIII, purification was improved about 2-fold over the previous steps. The mechanism of binding was not studied, but it seems reasonable that ATP bound to the enzyme might interact with calcium. On the other hand, data have been published [3, 4] which suggest that ATP induces conformational shifts in thymidine kinase. Such shifts might enhance enzyme-gel interactions.

Properties

The K_m value determined for thymidine in the present studies, viz. 3.72 ± 0.24 μM, was in the range of values reported previously for regenerating liver, viz. 5.6 ± 0.2 [3], but was lower than the value reported for calf thymus, viz. 50 μM [4]. Our estimate of the K_m value for ATP, viz. 0.29 ± 0.03 mM, was lower than estimates published by Bresnick et al. [3] for either high or low ATP levels. Furthermore, bimodality with respect to ATP which was previously reported for the *E. coli* [1] and regenerating rat liver enzymes [3] was not seen.

Although thymidine kinase from regenerating rat liver was inhibited by dTTP [7, 18], the nature of the inhibition was not well-characterized. With respect to the phosphate acceptor, dTTP was a competitive inhibitor for the kinase from calf thymus [4], *E. coli* [8], and one form of the Walker tumor [19]; in the aggregated form of the kinase from Walker tumor, dTTP inhibition was non-competitive [19]. With respect to the phosphate donor, dTTP inhibition was non-competitive for the enzyme from *E. coli* [1] and was complex for enzymes from calf thymus [4] and the Walker tumor [19]. In the presence of 400 μM thymidine, dTTP was a non-competitive inhibitor of ATP in our studies; but, when the thymidine concentration was lowered to 20 μM, then dTTP and ATP showed complex interactions. Okazaki and Kornberg [8] visualized two sites for triphosphate interactions, one an activator–inhibitor site,

the other a substrate site. If this analogy has application in the present study, when dTTP was in the activator–inhibitor site, the liver enzyme seemed to display substrate cooperativity which resulted in a considerable lowering of affinity for either substrate. Although concentration of Step VIII enzyme and adding bovine serum albumin to reaction mixtures appeared to stabilize catalytic activity, the possibility that enzyme inactivation influenced kinetic data must still be considered.

Molecular weight estimates for thymidine kinase from regenerating rat liver were 69 600 in this report and 80 600 in the study by Bresnick et al. [3]. In view of the procedure we used, our molecular weight estimate seems in reasonable accord with previous estimates for regenerating liver [3], for peak I enzyme from Yoshida sarcoma [6], and for the dimer form of the *E. coli* enzyme [2, 20].

ACKNOWLEDGEMENTS

We are grateful to Dr Edward Bresnick for recommending the procedure in which protein solutions were pre-treated with iodoacetate in order to minimize interference from Tris and mercaptoethanol during estimation of protein concentrations by the Lowry et al. [14] procedure.

REFERENCES

1 Okazaki, R. and Kornberg, A. (1964) J. Biol. Chem. 239, 269–274
2 Rohde, W. and Lezius, A. G. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1507–1516
3 Bresnick, E., Mainigi, K. D., Buccino, R. and Burleson, S. S. (1970) Cancer Res. 30, 2502–2506
4 Her, M. O. and Momparler, R. L. (1971) J. Biol. Chem. 246, 6152–6158
5 Weissman, S. M., Smellie, R. M. S. and Paul, J. (1960) Biochim. Biophys. Acta 45, 101–110
6 Hashimoto, T., Arima, T., Okuda, H. and Fujii, S. (1972) Cancer Res. 32, 67–73
7 Bresnick, E., Thompson, U. B., Morris, H. P. and Liebelt, A. G. (1964) Biochem. Biophys. Res. Commun. 16, 278–284
8 Okazaki, R. and Kornberg, A. (1964) J. Biol. Chem. 239, 275–284
9 Higgins, G. M. and Anderson, R. M. (1931) Arch. Pathol. 12, 186–202
10 Maley, G. F., Lorenson, M. G. and Maley, F. (1965) Biochem. Biophys. Res. Commun. 18, 364–370
11 Breitman, T. R. (1963) Biochim. Biophys. Acta 67, 153–155
12 Bruno, G. A. and Christian, J. E. (1961) Anal. Chem. 33, 1216–1218
13 Bush, E. T. (1963) Anal. Chem. 35, 1024–1029
14 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15 Albrecht, C. and Van Zyl, I. M. (1973) Exp. Cell Res. 76, 8–14
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17 Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
18 Ives, D. H., Morse, P. A., Jr. and Potter, V. R. (1963) J. Biol. Chem. 238, 1467–1474
19 Bresnick, E., Thompson, U. B. and Lyman, K. (1966) Arch. Biochem. Biophys. 114, 352–359
20 Iwatsuki, N. and Okazaki, R. (1967) J. Mol. Biol. 29, 139–154