Biochimica et Biophysica Acta, 527 (1978) 221—228

BBA 68604

PURIFICATION OF THYMIDINE PHOSPHORYLASE FROM HUMAN AMNIOCHORION

JOSEPH KUBILUS, LORETTA D. LEE and HOWARD P. BADEN

Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Mass. 02114 (U.S.A.)

(Received March 30th, 1978)
(Revised manuscript received July 24th, 1978)

Summary

Thymidine phosphorylase (thymidine : orthophosphate deoxyribosyltransferase, EC 2.4.2.4) has been purified 1500-fold from extracts of human amniochorion. The purified enzyme catalyzes the phosphorolysis of deoxythymidine and to a lesser extent deoxyuridine but not deoxycytidine nor uridine.

Discontinuous gel electrophoresis of the freshly purified enzyme shows a band containing 95% of the stainable protein. Gradient gel electrophoresis resolves the preparations into an active fraction with an apparent molecular weight of about 120 000 and a heavier less active or inactive fraction of about 180 000. Storage of the enzyme results in a decrease of the 120 000 dalton component, a loss in activity, and an apparent increase in the high molecular weight component. Sodium dodecyl sulfate gel electrophoresis shows only a single subunit of about 58 000 daltons which does not change on storage. These data are consistent with an active enzyme dimeric in structure which is capable of being converted to a less active form larger in molecular weight and possibly trimeric or tetrameric in structure.

Introduction

Pyrimidine nucleoside phosphorylases have been extensively purified from microorganisms [1—4] and also from several mammalian sources [5—9]. Uridine phosphorylases appear to catalyze the phosphorolysis of deoxyuridine, deoxythymidine, and uridine, while thymidine phosphorylases (thymidine : orthophosphate deoxyribosyltransferase, EC 2.4.2.4) are specific for the deoxyribose moiety and cause the phosphorolysis of thymidine and deoxyuridine but not uridine. In some mammalian tissues both enzymes have been identified and separated [10,11].

The purification of mammalian thymidine phosphorylase has been achieved [5—12]. In each case the purification achieved has been high (up to 1900-fold), but the homogeneities of the preparations have not been examined.

We report on the presence of pyrimidine nucleoside phosphorylase activity in human amniochorion and describe a rapid purification procedure for thymidine phosphorylase. Our examination of the purified preparation by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (SDS) and gradient gel electrophoresis is discussed.

Methods

Fresh placentas were collected and stored on ice. Within 48 h, the amniochorions were dissected from the body of the placenta, rinsed in cold water, and homogenized in an equal volume of ice-cold distilled water with a Waring Blendor. The homogenate was diluted with an equal volume of water and allowed to extract with stirring for 2 h at 4°C. Following centrifugation at 5000 × *g* for 20 min, the supernatant solution was lyophilized and stored at −20°C.

Purification. All procedures were done at 4°C. A 1% suspension of the lyophilized powder was made in 1 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA (pH 7.4). After 10-min stirring, the suspension was centrifuged at 5000 × *g* for 10 min. The supernatant solution was then treated with dropwise addition of 17.5 μl/ml of 2% protamine sulfate clear supernatant. The cloudy suspension which resulted was centrifuged at 7000 × *g* for 20 min and the pellet (which contained only trace activity) was discarded.

To the supernatant solution was added dropwise 22.5 μl/ml of 2% protamine sulfate. The resulting suspension was centrifuged as before and the pellet extracted overnight into 0.25 vol. 200 mM potassium phosphate buffer (pH 7.0). The solution was centrifuged at 5000 × *g* for 20 min. The pellet was discarded and the precipitate sedimenting between 20 and 35% $(NH_4)_2SO_4$ saturation was prepared. This precipitate was dissolved in 1 mM phosphate buffer, 1 mM EDTA, 1 mM dithiothreitol, 10 mM KCl and dialyzed extensively against the same buffer. This buffer was also the equilibration buffer and starting buffer for a 1.5 × 25 cm column of DEAE-cellulose. After washing, a linear gradient of 10—400 mM KCl was applied. Active fractions were combined and applied directly to a 0.9 × 7 cm column of hydroxyapatite (Bio-Rad HT) which had been equilibrated for 24 h in 2 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol. The absorbed sample was washed with starting buffer and then eluted with 20 mM phosphate buffer. All active fractions were combined. The resulting pool constituted our preparation of highly purified thymidine phosphorylase and was used for further experiments.

Gradient gel electrophoresis was performed on Pharmacia, PAA 4/30 gradient gels in a Pharmacia GE-4 gel electrophoresis apparatus. Prior to the run, highly purified enzyme was dialyzed into 5 mM potassium phosphate buffer (pH 7.4) and he gradient gel was pre-electrophoresed for 20 min at 70 V in the same buffer. Approx. 15 μg sample was then electrophoresed at 120 V for 15 h. Protein was then visualized by staining with Coomassie Brilliant Blue.

Discontinuous electrophoresis was performed on 5% polyacrylamide gels according to the method of Davis [13]. SDS gel electrophoresis was done according to the method of Neville [14]. Samples were prepared for SDS gel electrophoresis by heating at 50°C for 1 h in 1% SDS, 8 M urea, 0.1 M β-mercaptoethanol, followed by dialysis against electrophoresis buffer.

Enzyme assay. During purification, thymidine phosphorylase was routinely measured by the procedure of Yamada [10] in which the amount of thymine produced is determined spectrophotometrically by its absorbance at 295 nm in alkaline solution. Incubation mixtures consisted of 100 mM potassium phosphate, 5 mM β-mercaptoethanol and 3.3 mM thymidine in 1.5 ml. After 15 min incubation at 37°C, the reaction was stopped by the addition of 0.45 ml 2.1 M $HClO_4$. The mixture was chilled in an ice bath and then centrifuged. 1 ml clear supernatant solution was removed and made alkaline with 0.07 ml 9.6 M NaOH. The absorbance at 295 nm was then determined.

In the case of highly purified enzyme, addition of $HClO_4$ was omitted and the reaction was stopped by the addition of 0.60 ml 0.6 M NaOH and the absorbance read immediately at 295 nm. One unit of enzyme activity is that amount of protein which catalyzes the conversion of 1 μmol thymidine to thymine per h.

For the qualitative determination of cytidine, uridine, and deoxycytidine phosphorylase activities, a chromatographic assay was used. Reaction mixtures were identical to those in the spectrophotometric assay, but the reaction was allowed to proceed overnight. Incubation mixtures were deproteinized with $HClO_4$ as usual, but were then brought to neutral pH by addition of 5 M KOH. After centrifugation, suitable aliquots were removed and chromatographed on Eastman 6060 cellulose layers with distilled water. Nucleosides and free bases were visualized by viewing the chromatograms in ultraviolet light.

Results

Each amniochorion yielded about 1 g dry lyophilized material. Precipitation of the redissolved material by protamine sulfate occurred rapidly and the best purifications and yields were achieved when the preparation was centrifuged within 5 min of the addition of the complexing agent. The protamine precipitation was of particular value in that a 5-fold purification was achieved, the solution volume was reduced 4-fold and a significant amount of contaminating nucleic acid was removed. Prior to the treatment, the preparation had a 280 nm/260 nm ratio of 0.6 while the post-treatment value range from 1.25 to 1.35. Since prolonged exposure to protamine led to reduced yields, the activity-containing pellet was quickly dissolved and fractionated with $(NH_4)_2SO_2$.

Purification of thymidine phosphorylase as summarized in Table I yielded 1.35 mg protein and 10% of the original activity. Since the enzyme was close to 100% pure, it can be calculated that thymidine phosphorylase represent about 0.07% of the total water-soluble proteins of term amniochorion. Fig. 1 shows that, in DEAE-cellulose chromatography, the enzyme does not elute as a single protein peak, but as part of a shoulder. Only after hydroxyapatite chromatography does the enzyme appear as a discrete peak (Fig. 2). Monitoring of the

TABLE I

PURIFICATION OF THYMIDINE PHOSPHORYLASE FROM HUMAN AMINOCHORION

Units of activity are expressed as μmol thymidine converted per h. Protein was determined by the method of Lowry et al. [15].

Procedure	Units	Protein (mg)	Specific activity (units/mg)	Yield (%)
Lyophilized extract	4320	19 300	0.22	—
Protamine sulfate precipitation	2430	2 100	1.15	56
Amonium sulfate precipitation	1350	300	4.5	31
DEAE-cellulose chromatography	835	24	35	19
Hydroxyapatite chromatography	430	1.35	320	10

purification by electrophoretic analysis is shown in Fig. 3. A protein band which is not visible prior to DEAE-cellulose chromatography, is shown to be highly purified after the hydroxyapatite step. The highly purified enzyme consists of a major band which contains 95% of the stainable protein as determined by densitometer trace.

Gradient gel electrophoresis of the purified preparation revealed two protein bands as shown in Fig. 4. The band of higher R_F and therefore of lower molecular weight was the major component. After storage of the enzyme at 4°C a re-examination of the preparation showed that while the same two components were still present, the proportions had changed such that the ratio of minor band to major band had increased about 3-fold. Activity measurements showed that storage resulted in a decrease in activity of over 50%.

Electrophoresis of the purified enzyme in the presence of SDS shows a single

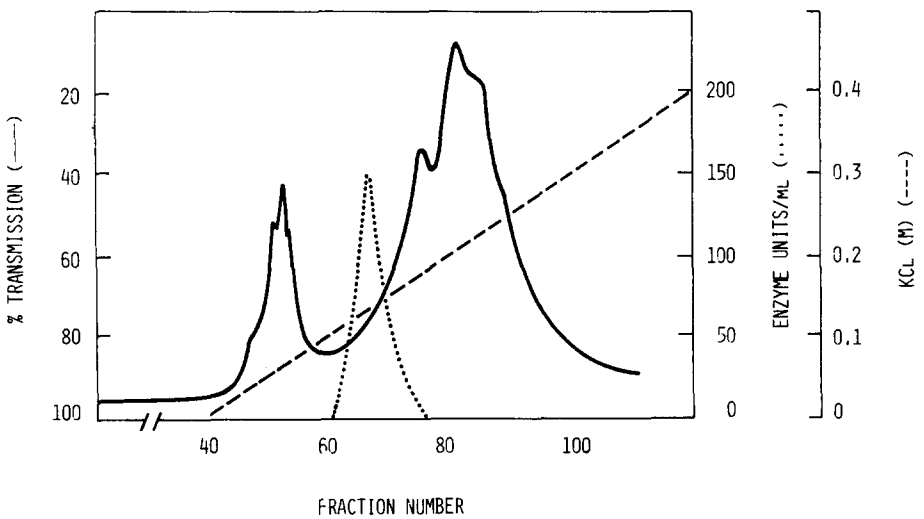

Fig. 1. Chromatography of partially purified thymidine phosphorylase on DEAE-cellulose. Percent transmission is measured at 280 nm. 300 mg enzyme was applied at 6 ml per h in 1 mM phosphate buffer (pH 7.4), 10 mM KCl, 1 mM dithiothreitol, 1 mM EDTA. The enzyme was eluted with a linear gradient of KCl.

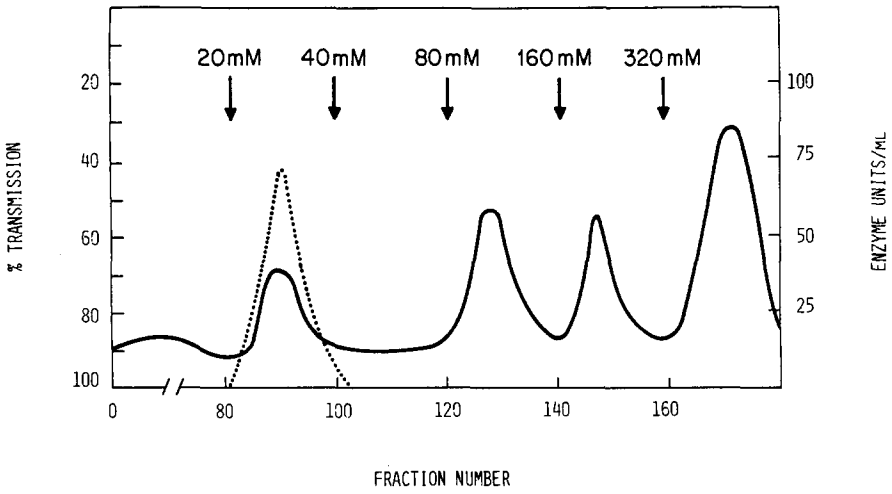

Fig. 2. Chromatography of thymidine phosphorylase on hydroxyapatite. The enzyme as eluted from DEAE-cellulose was applied directly to the column and consisted of about 24 mg protein. Elution was achieved by washing with stepwise increases of potassium phosphate in 1 mM dithiothreitol, 1 mM EDTA at 1 ml/h.

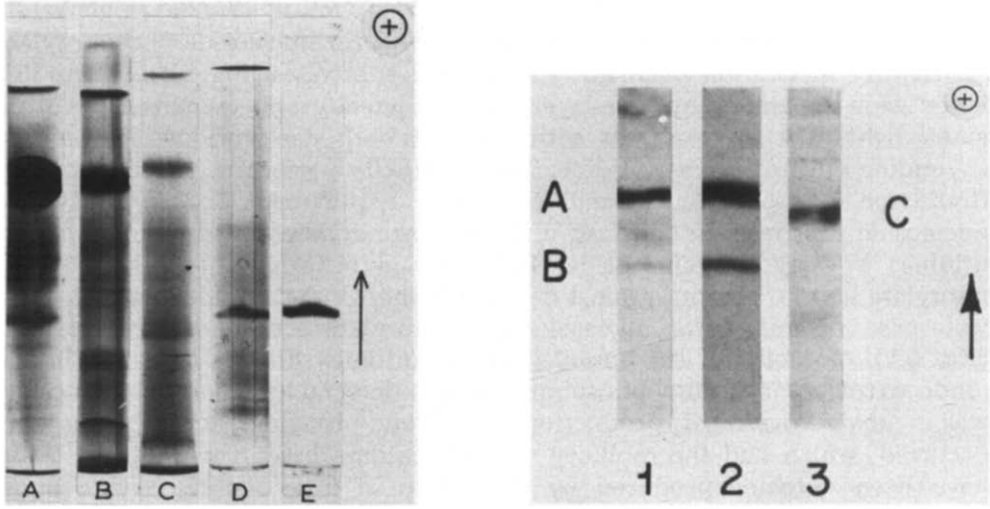

Fig. 3. Purification of thymidine phosphorylase as monitored by discontinuous gel electrophoresis. After each purification step the preparation was electrophoresed in the Tris/glycine-HCl system of Davis [13]. A, crude enzyme; B, protamine sulfate precipitate; C, $(NH_4)_2SO_4$ precipitate; D, DEAE-cellulose fraction; E, hydroxyapatite fraction.

Fig. 4. Gradient gel electrophoresis at pH 7.4 in a polyacrylamide gradient of 4—30%. (1) Freshly purified thymidine phosphorylase. (2) Purified thymidine phosphorylase after storage at 4°C for 1 month. (3) Bovine serum albumin dimer. Densitometer tracings of gels 1 and 2 showed that the ratio of B and A was three times higher in gel 2 than in gel 1.

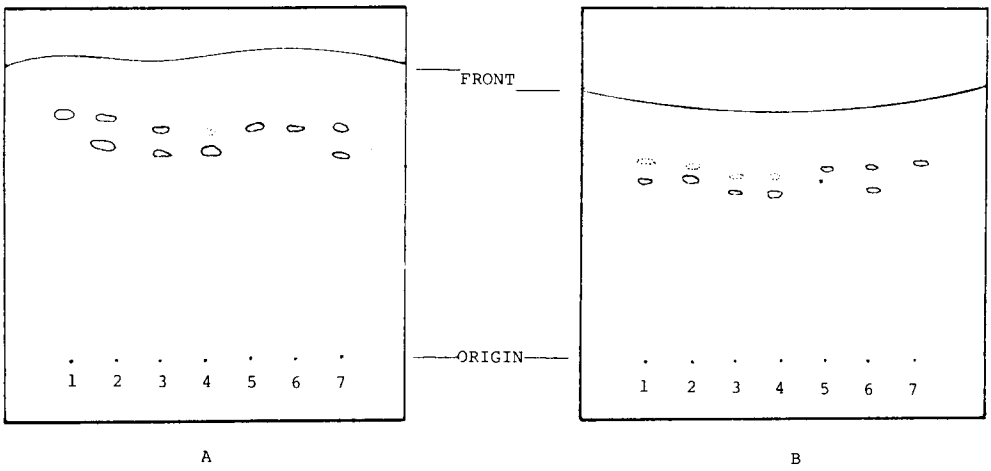

Fig. 5. Thin-layer chromatography of reaction products from 16-h incubations of various nucleosides with thymidine phosphorylase. (A) All incubations contained crude enzyme. 1, cytidine; 2, cytidine co-chromatographed with cytosine; 3, deoxycytidine; 4, deoxyuridine; 5, deoxyuridine co-chromatographed with uracil; 6, uridine; 7, uridine co-chromatographed with uracil. (B) All incubations contained pure enzyme. 1, thymidine; thymidine co-chromatographed with thymine; 3, deoxyuridine; 4, deoxyuridine co-chromatographed with uracil; 5, uridine; 6, uridine co-chromatographed with uracil; 7, deoxycytidine.

band. A molecular weight of 55 000—60 000 was calculated (in comparison to standard proteins) for the dissociated enzyme. It is important to note that SDS gel electrophoresis of the stored enzyme also showed a similar single band.

Purified thymidine phosphorylase was tested for nucleoside phosphorylase activity by incubation overnight with various substrates (Fig. 5B). Pyrimidine bases were separated by thin-layer chromatography and visualized by ultraviolet light. The enzyme was active against both deoxyuridine and deoxythymidine but not against uridine. This specificity pattern is characteristic for thymidine phosphorylases, and reflects a requirement for a deoxyribonucleoside substrate. In contrast, uridine phosphorylases are also active against uridine, a 2′-hydroxynucleoside. Purified amniochorionic thymidine phosphorylase had no activity against deoxycytidine. Crude amniochorion extracts were also examined for nucleoside phosphorylase activity by this method (Fig. 5A). No activity was observed against uridine, showing that even in our crude extracts, no uridine phosphorylase was detectable. When deoxycytidine was incubated overnight, production of a second ultraviolet-absorbing material occurred, which had the mobility of a pyrimidine base. This material could have been cytocine produced by the action of a second nucleoside phosphorylase, or uracil produced by the deamination of deoxycytidine to deoxyuridine followed by phosphorolysis by thimidine phosphorylase.

Discussion

The ability to effect the enzymatic phosphorolysis of thymidine is widespread among bacteria and mammalian tissue. An enzyme responsible for this ability is thymidine phosphorylase. Bacterial enzymes have been highly purified

from both *Escherichia coli* and *Salmonella typhimurium* [1—3]. Mammalian enzymes have been purified from rabbit liver, rat liver human spleen and human leukocytes. The specific activities of the purified mammalian enzymes have been lower than that of the placental enzyme with the exception of the spleen enzyme which was about 1500 μmol/h per mg protein. Variation in specific activities may be due to incomplete purification, or to differences in turnover number of enzymes purified from different tissues of a number of organisms.

Thymidine phosphorylases from a number of sources have similar properties and many have a molecular weight of 100 000—120 000, all have a similar pH optima for the phosphorylation and most possess deoxyribosyltransferase activity. They are strongly anionic and form insoluble complexes with cationic species such as protamine and streptomycin, bind strongly to DEAE-cellulose and DEAE-cellulose and DEAE-Sephadex and can be extensively purified on hydroxyapatite. The enzyme which we have purified from human amniochorion shares these characteristics and has been purified by a combination of techniques found useful for other thymidine phosphorylases.

Because thymidine phosphorylases have not as a rule been examined for homogeneity and because of variation in specific activities observed for the enzyme, we carefully examined the purity of our preparation. Freshly purified amniochorion enzyme was assessed by three different electrophoretic procedures. SDS gel electrophoresis showed a basic subunit of about 58 000 which is the molecular weight of the denatured and dissociated enzyme. Electrophoresis in a gradient gel system done under conditions of pH and ionic strength which stabilize the activity of the enzyme shows a 'native' molecular weight of 110 000—120 000, with a minor second species at a molecular weight of approx. 180 000. Electrophoresis in the non-dissociating conditions of a Tris/glycine-HCl system also show a major band with trace contaminants.

From the SDS gel electrophoretic data it could be concluded that the active enzyme consisted of a dimer composed of two subunits of about 55 000—60 000 while the higher molecular weight component consists of three such subunits. Blank and Hoffee [3] have proposed a dimeric structure for the thymidine phosphorylase of *S. typhimurium* which has a native molecular weight of 100 000—110 000 and an SDS-dissociated molecular weight of 47 000.

After storage at 4°C for several weeks, our purified enzyme preparation lost activity. Electrophoretic analysis showed a decrease in the amount of 110 000——120 000 dalton species and an increase in the amount of 180 000 dalton bands. We concluded that most of the activity was associated with the 120 000 dalton species while the 180 000 dalton component was inactive or less active. Although the formation of trimer is associated with loss of activity, it does not account for all of the observed loss.

Similar behavior has been observed for a uridine phosphorylase purified from rat liver [10,16], in that loss of activity and change of electrophoretic pattern is observed after storage. These changes have been associated with exposure to Tris buffer. Amniochorionic thymidine phosphorylase is purified by procedures which do not involve exposure to Tris buffer, so the changes which we observe, although quite similar, are caused by a different agent. A similar

effect also occurs when *E. coli* thymidine phosphorylase is exposed briefly to ultraviolet light. There is a loss of activity and generation of material higher in molecular weight than the native enzyme [2]. These results suggest that the light-induced denaturation of the *E. coli* enzyme may enhance the formation of higher polymers. Although we have not determined the reason for the instability of the human enzyme during storage, we must point out that no precautions were taken against light exposure, and we cannot rule out that light plays some role in the denaturation process. If other thymidine phosphorylases are as unstable on storage as the amniochorionic enzyme, it may help to explain why homogeneous preparations of this enzyme have not been reported.

Acknowledgement

This work was supported by N.I.H. Grant 5 R01 H 07461.

References

1 Razzell, W.E. and Khorana, H.G. (1958) Biochim. Biophys. Acta 28, 562—566
2 Voytek, P. (1975) J. Biol. Chem. 250, 3660—3665
3 Blank, J.G. and Hoffee, P.A. (1975) Arch. Biochem. Biophys. 168, 259—265
4 Schwartz, M. (1971) Eur. J. Biochem. 21, 191—198
5 Friedkin, M. and Roberts, D. (1954) J. Biol. Chem. 207, 245—256
6 Zimmerman, M. (1964) J. Biol. Chem. 239, 2622—2627
7 Lyon, G.R. (1968) Biochim. Biophys. Acta 159, 38—49
8 Krenitzky, T.A. (1968) J. Biol. Chem. 243, 2871—2875
9 Gallo, R.C. and Breitman, T.R. (1968) J. Biol. Chem. 243, 4943—4951
10 Yamada, E.W. (1968) J. Biol. Chem. 243, 1649—1655
11 Kraut, A. and Yamada, E.W. (1971) J. Biol. Chem. 246, 2021—2030
12 Gallo, R.C. and Brietman, T.R. (1968) J. Biol. Chem. 243, 4936—4942
13 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
14 Neville, Jr., D.M. (1971) J. Biol. Chem. 246, 6328—6334
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Bose, R. and Yamada, E. (1974) Biochemistry 13, 2051—2056