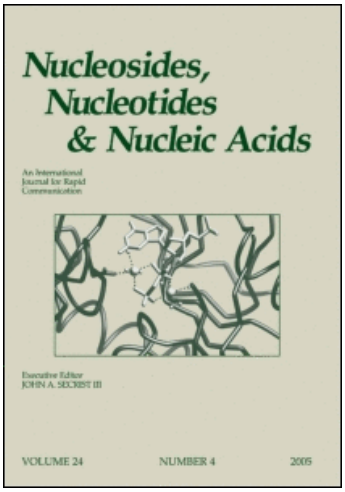

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Liya Wang[a]; Staffan Eriksson[a]
[a] Department of Anatomy, Physiology and Biochemistry, Section of Veterinary Medical Biochemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, Uppsala, Sweden

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5-BROMOVINYL 2′-DEOXYURIDINE PHOSPHORYLATION BY MITOCHONDRIAL AND CYTOSOLIC THYMIDINE KINASE (TK2 AND TK1) AND ITS USE IN SELECTIVE MEASUREMENT OF TK2 ACTIVITY IN CRUDE EXTRACTS

Liya Wang and Staffan Eriksson

Department of Anatomy, Physiology and Biochemistry, Section of Veterinary Medical Biochemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, Uppsala, Sweden

□ *Mitochondrial thymidine kinase (TK2) is responsible for phosphorylation of thymidine and deoxycytidine and plays a crucial role in mitochondrial DNA precursor synthesis. TK2 is expressed in all tissues at low levels complicating accurate determinations, especially in tissues with high cytosolic thymidine kinase (TK1) activity. Recently, 5-bromovinyl 2′-deoxyuridine (BvdU) at 0.2 μM was used to measure TK2 activity selectively. BvdU phosphorylation by pure human TK2 and TK1 was tested here, and the ratio of BvdU phosphorylation by TK2/TK1 was 91 at 0.2 μM but was 500 at 2.5 μM. Therefore, for reliable measurement of TK2 activity higher BvdU concentration should be used.*

Keywords Thymidine kinase 2; thymidine kinase 1; activity determination; bromovinyl deoxyurindine

INTRODUCTION

The level of TK2 activity has been associated with mitochondrial DNA (mtDNA) depletions in patients with genetic defect of the TK2 gene or AIDS patients who underwent chemotherapy with nucleoside analogs. However, measurement of TK2 activity in tissue extracts is complicated by the low level of TK2 and other interfering enzymes such as TK1. TK1 phosphorylates dThd and deoxyuridine (dUrd), which are substrates for TK2, and has a 10-fold higher catalytic efficiency (k_{cat}/K_m) as compared with TK2, and therefore, interferes with TK2 activity measurement.[1] Recently, BvdU was suggested as alternative substrate for TK2 at submicromolar concentration to measure TK2 activity in crude extracts.[2]

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Address correspondence to Staffan Eriksson, Department of Anatomy, Physiology and Biochemistry, Section of Veterinary Medical Biochemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, P.O. Box 575, SE-751 23 Uppsala, Sweden. E-mail: staffan.eriksson@afb.slu.se

In this work, we studied the phosphorylation of BvdU by pure human recombinant TK2 and TK1 and found that for selective TK2 determinations 2.5 μM BvdU was optimal. We found that BvdU was rapidly degraded in certain tissue extracts by thymidine phosphorylase (TP), thus, interfering with TK2 activity measurement. By using a TP specific inhibitor we could overcome this problem and total TK and TK2 activities in mouse tissue extracts were determined and showed ~40-fold variation in TK2 levels.

MATERIALS AND METHODS

[Methyl-^{3}H]-thymidine ([^{3}H]-dThd, 20 Ci/mmol) was from Perkin Elmer(Perkin Elmer Sverige AB, Upplands Väsby, Sweden) and [5′-^{3}H] 5-bromovinyl-2′-deoxyuridine ([^{3}H]-BvdU, 10 Ci/mmol) was from Moravek Biochemical Inc. (Brea, CA, USA). Nonradioactive nucleosides and nucleotides were from Sigma (Sigma-Aldrich Sweden AB, Stockholm, Sweden).

Recombinant human TK2 and TK1 were expressed, purified and assayed as described earlier.[3] TK (TK1 and TK2) activity in crude extracts was determined using [^{3}H]-dThd or [^{3}H]-BvdU as substrate as previously described.[1,2]

RESULTS AND DISCUSSION

BvdU Phosphorylation by TK2 and TK1

BvdU has been shown to be a substrate for TK2,[3,4] but no study with TK1 have been published. Using pure recombinant human enzymes we studied BvdU phosphorylation by both TK2 and TK1 and showed that BvdU phosphorylation by TK2 followed Michaelis-Menten kinetic and the K_m and V_{max} values were 2.5 μM and 714 nmol/min/mg, respectively, which gave an efficiency (V_{max}/K_m) ~70% of that of dThd. The phosphorylation by TK1 was not saturated with BvdU concentration up to 160 μM.

As shown in Table 1 the highest ratio of phosphorylation by TK2 compared to TK1 was with 2.5 μM BvdU. At concentrations lower or higher the TK2/TK1 ratio decreased. These results suggest that using 2.5 μM BvdU in assays to measure TK2 activity is most selective.

BvdU Phosphorylation and Phosphorolysis in Crude Tissue Extracts

A concern with BvdU as selective substrate for TK2 activity determination in crude extracts was the low BvdU concentration proposed by Franzolin et al.[2] At submicromolar concentration (0.2 μM) the activity with pure TK2 was only about 7% of the V_{max} value and this may compromise the measurement in tissues where the TK2 activity is low. The TK activities in crude extracts were assayed with different BvdU concentrations (Table 2).

TABLE 1 The ratio of BvdU phosphorylation by TK2 and TK1*

BvdU (μM)	TK2/TK1
0.2	91
2.5	500
10	178
40	54

*Pure recombinant human TK1 and TK2 were used in the assays using [^{3}H]-BvdU as substrate at concentration range of 1 to 160 μM. With TK2 the substrate saturation curve followed Michaelis-Menten equation while with TK1 the activity was linear in the concentration range and did not reach saturation. The ratio was calculated from the activity at each concentration for TK1 and TK2.

With 0.2 μM BvdU the TK activities were below 1 pmoles/min/mg. With higher BvdU concentration the TK activities were also higher. However, TK activity in liver extracts determined with BvdU did not correlate with assays using [^{3}H]-dThd as substrate (Table 2). Analysis of the reaction products showed that [^{3}H]-BvdU was degraded to 5-bromovinyl uracil and [^{3}H]-deoxyribose 1-phosphate (data not shown) and the labelled deoxyribose-1-phosphate retained on the DE81 filter that led to higher TK activity. BvdU is known to be a substrate for TP and liver has high level of TP activity,[5] which explained the high activity with BvdU. When 6-amino-5-chlorouracil, a TP specific inhibitor, was included in the assay the TK activities measured with BvdU were comparable to those with [^{3}H]-dThd (Table 2).

TK2 Activities in Mouse Tissues Determined by Using Either dThd or BvdU as Substrate

Determination of TK2 activity in mouse tissue extracts using BvdU in the presence of 6-amino-5-chlorouracil was done and compared to that when TK2 activity was measured with dThd (Table 3). The TK2 levels determined with BvdU were low as compared with the TK2 activities estimated from

TABLE 2 TK activity in mouse liver and spleen extracts (pmol/min/mg)*

Substrate	BvdU				dThd	BvdU+TPI	
(μM)	0.2	2.5	10	40	100	2.5	10
Liver	0.65 ± 0.1	4.8 ± 0.2	13.7 ± 0.5	47.9 ± 2.1	4.1 ± 0.2	0.7 ± 0.1	1.2 ± 0.1
Spleen	0.52 ± 0.1	1.7 ± 0.1	4.2 ± 0.2	5.8 ± 1.0	256 ± 10	1.5 ± 0.1	2.1 ± 0.15

*Assays were performed with mouse liver and spleen extracts using either 3H-BvdU or 3H-dThd as substrate at various concentration. The experiments were done in duplicates of extracts from three individual mice. The results are mean ± SE.

TPI, thymidine phosphorylase inhibitor, 6-amino-5-chlorouracil at 1 mM.

TABLE 3 TK2 activities in mouse tissue extracts*

	Total TK	TK2	
	dThd	dThd	BvdU
Liver	3.8 ± 0.3	3.8	0.73
Spleen	211 ± 88	#	1.50
Lung	3.3 ± 0.3	3.3	0.64
Kidney	2.2 ± 0.7	2.2	0.64
Cardiac	2.4 ± 0.4	2.4	0.32
Brain	13.8 ± 1.0	13.8	2.32
Pancreas	2.6 ± 0.3	2.6	0.48
Testis	14.6 ± 0.8	14.6	1.63
Biceps	0.4 ± 0.2	0.4	<0.01
Vastus	0.7 ± 0.4	0.7	<0.01
Semitendinosus	0.5 ± 0.2	0.5	<0.01
Brown fat	5.7 ± 0.7	5.7	1.32
Diaphragm	0.8 ± 0.1	0.8	0.13
Adipose	5.8 ± 1.0	5.8	0.62

*Total TK activity was determined by using 100 μM [^{3}H]-dThd. TK2 activity was estimated from assays with [^{3}H]-dThd in the presence of excess dCyd (1 mM). If the inhibition by dCyd is > 50% inhibition then the total TK activity equals TK2 activity. If the inhibition by dCyd is <40% the level of TK1 in the sample is too high and TK2 activity cannot be estimated, for example, in the spleen. In such case TK2 activity can be determined by using [^{3}H]-BvdU (2.5 μM) in the presence of 1 mM 6-amino 5-chlorouracil.

#TK2 activity could not be estimated due to the presence of high TK1 activity.

assays with dThd due to the lower V_{max} value of BvdU and low BvdU concentration used. With 2.5 μM BvdU as substrate the TK2 activity in spleen extract could be determined (1.5 pmoles/min and mg which corresponds to approx. 4 pmoles dTMP formed/min and mg) which was not possible earlier because of the high level of TK1.[1] Brain, testis, and spleen have the highest TK2 level; liver, lung, kidney, cardiac pancreas brown fat, and adipose tissues have 3- to 5-fold lower TK2 activity; skeletal muscle—biceps, vastus, and intermedius semitendinosus have the lowest TK2 activity which was measurable only with dThd but not with 2.5 μM BvdU and these results were overall comparable to those reported earlier.[1]

CONCLUSION

Tritiated thymidine can be used to determine TK2 and TK1 activities in tissue extracts when the assays are conducted in the absence and presence of excess dCyd to inhibit selectively TK2 activity. In case of high TK1 activity, for example, in spleen extracts, BvdU at 2.5 μM concentration together with a TP inhibitor provides an alternative and reliable substrate for TK2 activity measurement.

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