THYMIDINE KINASE ACTIVITY IN RYE CHLOROPLASTS

Tomasz GOLASZEWSKI, Marta RYTEL, Jerzy ROGOZIŃSKI* and Jan W. SZARKOWSKI

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02–532 Warszawa, Rakowiecka 36, Poland

Received 2 September 1975

1. Introduction

Thymidine kinase (ATP, thymidine 5-phosphotransferase, EC 2.7.1.75) activity has been studied in a variety of biological systems. However, neither the subcellular distribution of the enzyme, particularily in plants, nor its involvement in DNA synthesis has been fully elucidated [cf.1].

Here we report on the occurrence of thymidine kinase in chloroplasts isolated from rye (*Secale cereale*) seedlings.

2. Materials and methods

Rye, var. 'Dankowskie', grains were surface sterilized with 0.1 % (w/v) $HgCl_2$ solution in water and rinsed with water. The grains were imbibed in tap water for 2 h and then grown on sterilized lignine moistened with water for a given period (see Results) at room temperature under a normal light regime.

The leaves were homogenized in a mortar in 0.15 M phosphate buffer, pH 7.3, containing 0.3 M sucrose, 0.01 M $MgCl_2$ and 0.01 M KCl. The homogenate was filtered through a nylon tissue and subjected to differential centrifugation. After pelleting nuclei and cell debris at 500 *g* for 10 min, the resultant supernatant was centrifuged at 1000 *g* for 14 min to sediment crude chloroplasts.

Crude chloroplasts were suspended in a fresh portion of the grinding medium and pelleted at 3000 *g* for 20 min. The pellet obtained, suspended in a small volume of the same medium, was layered on top of a discontinuous sucrose density gradient consisting of three layers: 1.0 M, 1.5 M and 2.0 M sucrose in 0.15 M phosphate buffer, pH 7.3. After centrifugation at 25 000 *g* for 20 min, the green pellet band, sedimented in the 1.5 M sucrose layer, was collected. It was suspended in 50 ml of the grinding medium and centrifuged at 3000 *g* for 20 min to sediment purified chloroplasts [2].

The chloroplast preparations were examined under the light microscope. The chemical analysis showed that the chlorophyll/protein ratio of a given chloroplast preparation obtained after the last purification step was approximately the same as that found for chloroplasts collected from the sucrose density gradient.

In some experiments chloroplasts suspended in water were sonicated with a MSE Ultrasonic Disintegrator at 2°C for 3 min (with 30 sec intervals after every 60 sec) at 20 000 cycles/sec. The sonicate was subjected to differential centrifugation; three subfractions were obtained: 20 000 *g* pellet obtained after centrifugation for 20 min; 144 000 *g* pellet obtained after centrifugation for 2 h, and the 144 000 *g* supernatant.

Post-ribosomal supernatant, referred to as cytosol, was obtained from leaf homogenates by centrifuging the postmitochondrial supernatant at 105 000 *g* for 2 h.

Thymidine kinase activity was measured as described by Berk et al. [3], except that the reaction mixture contained 9 μM 6-[^{3}H]thymidine (specific activity 22.0 Ci/mmole). 100 μg of protein to be checked for enzyme activity was added; to the control

* Agriculture Academy, 02–532 Warszawa, Rakowiecka 26/30, Poland.

North-Holland Publishing Company – Amsterdam

sample the same amount of bovine serum albumin was added. The optimal protein concentration for the assay was established previously [4]. The radioactivity of dTMP bound to DEAE filter paper discs was counted in a Packard Liquid Scintillation Spectrometer, using PPO-POPOP-toluene mixture as a scintillation liquid. cpm Values were converted into pmol of dTMP on the basis of counts of 6-[^{3}H] thymidine measured under conditions and geometry identical to those used in counting the reaction product. Activity of non-specific nucleoside phosphotransferase was measured under similar conditions, except that AMP, instead of ATP, was used as a phosphate donor. The activity of both thymidine phosphorylating enzymes is expressed in pmol of dTMP formed under the experimental conditions used (during 15 min at 37°C) per mg of protein.

To prove that the reaction product was dTMP, the reaction mixture (after stopping the reaction by adding cold trichloracetic acid to 5% final concentration) was subjected to paper chromatography as described previously [4]. A major part of radioactivity was recovered in a spot co-migrating with that of the 5′-dTMP standard.

Protein was estimated by the method of Lowry et al. [5]. Chlorophyll content in chloroplast preparations was determined according to Arnon [6].

3. Results

Purified chloroplasts isolated from rye seedling leaves exhibited activities of the thymidine phosphorylating enzymes, thymidine kinase, and non-specific nucleoside phosphotransferase. The specific activity of both enzymes changed during the early stage of plant growth.

As can be seen from table 1, the highest specific activity of thymidine kinase was observed in chloroplasts isolated from 10-day-old seedlings; a significant drop in specific activity of the enzyme occurred in chloroplasts derived from older plants. The highest level of nucleoside phosphotransferase activity was found in chloroplasts isolated from 7-day-old seedlings. A relatively low activity of the latter enzyme was found in chloroplasts from 10-day-old plants.

Both enzyme activities were also present in the cytosol prepared from rye leaves. The specific activities, however, were markedly lower. Concomitantly with an increase in activity of chloroplast thymidine kinase, some decrease in the level of the cytosolic enzyme was experienced.

To check whether or not the 'developmental' changes in chloroplast thymidine kinase activity (table 1) are due to differences in enzyme stability, tests summarized in table 2 were carried out. It may

Table 1
Thymidine phosphorylating enzyme activities in growing rye seedlings at subcellular level

Plant age	Thymidine kinase (spec. act. pmol of dTMP/mg of protein)		Nucleoside phosphotransferase (spec. act. pmol of dTMP/mg of protein)	
(days)	chloroplasts 1	cytosol 2	chloroplasts 3	cytosol 4
4	405 ± 15	101 ± 7	77 ± 6	5
7	514 ± 19	198 ± 5	151 ± 7	10
10	663 ± 4	50 ± 7	36 ± 3	18
12	558 ± 29	86 ± 7	4 ± 2	28
16	94 ± 11	–	–	–

Chloroplasts,purified according to Mani and Zalik [2], were homogenized in water with a glass homogenizer. Preparation of cytosol, see Materials and methods. Thymidine kinase activity was measured under conditions described by Berk et al. [3], nucleoside phosphotransferase activity was measured under the same conditions, except that AMP was used as a donor of phosphate groups. Protein was estimated by the method of Lowry et al. [5]. Data in columns 1,2 and 3 represent the mean of 4–6 experiments ± S. E. M.; data in column 4 represent the mean of 3 experiments.

Table 2
Stability of thymidine kinase in chloroplasts isolated from rye seedlings of various ages

Plant age	% of activity retained[a]	
	preincubation period	
(days)	60 min	120 min
4	72	60
7	68	35
10	56	10
12	74	15
16	90	63

Chloroplast homogenates to be used in assays for thymidine kinase stability were preincubated for a given period at 37°C in a mixture containing 0.002 M dithiothreitol, 0.010 M $MgCl_2$ and 0.16 M Tris–HCl buffer, pH 7.6. Thymidine kinase activity was measured according to Berk et al. [3]. The figures presented are averages from several experiments.
[a] 100% = thymidine kinase activity found in chloroplast preparations (obtained from seedlings of respective age) not preincubated before the assay.

be seen that the stability of chloroplast thymidine kinase was different in chloroplast preparations obtained from seedlings of different ages. It seems, however, that 'developmental' changes of thymidine kinase activity in chloroplasts (table 1) are not due to differential rates of enzyme inactivation (i.e. artefacts).

Table 3
Distribution of thymidine kinase activity within rye chloroplasts

Chloroplast subfraction	Spec. act. (pmol of dTMP per mg of protein)	% of total activity
1. 20 000 *g* pellet	30[a]	2
2. 144 000 *g* pellet	456[a]	25
3. 144 000 *g* supernatant	448 ± 6	73

Chloroplasts were isolated from 10-day-old seedlings and purified according to Mani and Zalik [2]. The chloroplast preparation was sonicated as described in Materials and methods and subjected to differential centrifugation. Data in lines 1 and 2 represent the mean of 2 determinations, those in line 3 represent the mean of 4 determinations ± S.E.M.
[a] Pretreatment of pellets with Triton X-100 did not enhance the activity.

The distribution of thymidine kinase within the chloroplasts was also examined. Table 3 indicates that a major part of enzyme activity was detected in the supernatant obtained after centrifugation of chloroplast sonicates at 144 000 *g* ; only a small part of total activity was found in the pellet sedimenting at 20 000 *g*. In the latter case the specific activity of thymidine kinase was 15-fold lower than that of the enzyme(s) present in the two other chloroplast subfractions.

The relative proportions of total activities of thymidine kinase and nucleoside phosphotransferase present in the chloroplasts and in the cytosol prepared from 10-day-old seedling leaves were compared. Table 4 shows that the total activity of thymidine kinase in cytosol is nearly 3-fold higher than that in the chloroplasts. On the contrary, only a very small portion of the total activity of nucleoside phosphotransferase was found in chloroplasts.

4. Discussion

Little is known about the localization of thymidine kinase in cells of higher plants although the occurrence of the enzyme in this group of organisms is firmly established [cf.7]. Appearance of thymidine kinase activity in developing plants after onset of seed germination has been reported [8–10] and compartmentation of the enzyme in cells of germinating plants has been considered [4].

Here we report that both thymidine kinase and nucleoside phosphotransferase occur in rye chloroplasts. The specific activities of both enzymes change

Table 4
Relative amounts of thymidine phosphorylating enzymes in chloroplasts and cytosol prepared from 10-day-old rye seedling leaves

Subcellular fraction	Total activities (pmoles of dTMP)	
	Thymidine kinase	Nucleoside phosphotransferase
Chloroplasts	1439	73
Cytosol	3660	1314

For details of enzyme assays, see Materials and methods. The data represent the mean of two experiments.

during plant growth. The highest specific activity of thymidine kinase was observed in chloroplasts isolated from 10-day-old seedlings. A significant decrease in enzyme activity was observed in chloroplasts obtained from older plants. The specific activity of nucleoside phosphotransferase, which was markedly lower during the plant growth period studied, reached its maximum in chloroplasts originating from 7-day-old seedlings. Thus, with chloroplasts originating from 10-day-old seedlings the activity of nucleoside phosphotransferase may contribute to the thymidine kinase activity reported only to a small extent.

Plant thymidine kinases are known to be extremely labile [7]. Still, a peak of thymidine kinase activity in chloroplasts from the 10-day-old rye seedlings was observed which does not seem to be due to differential inactivation of the enzyme (table 2).

In view of the level of thymidine kinase activity in rye cytosol it seems unlikely that the enzyme found in chloroplasts constitutes merely a contaminant of cytosolic origin. A relatively large proportion of the total activity of thymidine kinase found in chloroplasts as compared to that in the cytosol prepared from the plants of the same age (10-days) is noteworthy in this respect.

As far as the suborganellar distribution of thymidine kinase activity is concerned, the results support the assumption that the enzyme associated with rye chloroplasts is localized mainly in the matrix of the organelles.

The role of thymidine kinase in DNA synthesis is not clear, as yet. Recently, thymidine kinase activity in chloroplasts of the alga *Chlamydomonas reinchardtii* was reported and an active role of the enzyme in chloroplast DNA synthesis during vegetative growth has been postulated [11]. Besides, thymidine kinase bound with a subcellular fraction comprising chloroplasts and mitochondria isolated from *Acetabularia thallus* has been described [12]. Our results indicate that rye chloroplasts also contain this enzyme.

Compartmentation of thymidine kinases in various mammalian cells has been reported earlier and mitochondrial enzymes, along with cytosolic thymidine kinases, are known [cf.3]. In view of data reported here and those quoted above concerning thymidine kinases in chloroplasts of algae [11,12], one may assume that compartmentation of the enzyme is a rather common phenomenon, not limited to some mammalian cells. Thus, the possibility should be taken into account that organellar (i.e. plastidial and mitochondrial, as opposed to cytosolic) thymidine kinases are of some importance in extranuclear DNA synthesis, and perhaps in organellogenesis.

Acknowledgements

Investigation supported by the Polish Academy of Sciences within project 09.3.1.

References

[1] Olsen, I. and Harris, G. (1975) Biochem. J. 146, 489–496.
[2] Mani, R. S. and Zalik, S. (1970) Biochim. Biophys. Acta 200, 132–137.
[3] Berk, A. J., Mayèr, B. J. and Clayton, D. A. (1973) Arch. Biochem. Biophys. 154, 563–565.
[4] Gołaszewski, T. and Szarkowski, J. W. (1974) Internat. J. Biochem. 5, 597–599.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[6] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[7] Brunori, A., Hotta, Y. and Stern, H. (1974) Plant Cell Physiol. 15, 421–427.
[8] Wanka, F., Vasil, I. K. and Stern, H. (1964) Biochim. Biophys. Acta 85, 50–59.
[9] Wanka, F. and Walboomers, J. M. (1966) Z. Phlanzenphysiol. 55, 458–465.
[10] Schwarz, O. J. and Fites, R. C. (1970) Phytochem. 9, 1899–1905.
[11] Chiang, K. S., Eves, E. and Swinton, D. (1975) Develop. Biology 42, 53–63.
[12] Bannwarth, H. (1975) Protoplasma 83, 169–170.