Note

Occurrence and Subcellular Distribution of Thiamine Pyrophosphokinase Isozyme in *Euglena gracilis*Shigeru SHIGEOKA, Toshio ONISHI,
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The biosynthetic pathways of thiamine pyrophosphate from thiamine occur in two distinct types: one by a one-step reaction catalyzed by thiamine pyrophosphokinase (EC 2.7.6.2) in yeast¹⁾ and the other by two steps catalyzed by thiamine kinase (EC 2.7.1.89) and thiamine monophosphate kinase (EC 2.7.4.16) in *Escherichia coli*.²⁾ *Euglena gracilis* absolutely requires thiamine for growth³⁾ and contains an active transport system for thiamine.⁴⁾ When thiamine and its phosphate esters were fed to thiamine-deficient cells, these compounds accumulated in the cells in the form of free thiamine, but not in the ester forms.³⁾ These results suggested that *E. gracilis* has an enzyme system that catalyzes the phosphorylation of thiamine to thiamine pyrophosphate to supply the coenzyme for its dependent enzymes. However, there is no information on the conversion of thiamine to thiamine pyrophosphate and its location. This paper reports the occurrence and subcellular distribution of thiamine pyrophosphokinase isozymes in *Euglena gracilis*.

E. gracilis thiamine-sufficient cells were cultured in 150 ml of Koren-Hutner⁵⁾ medium containing 100 μ g of thiamine for 5 days at 26°C under illumination (3,000 lux). *Euglena* cells (0.5 g wet weight) were harvested, washed twice with distilled water, suspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication (10 kHz, 3 min). The supernatant obtained by centrifugation (10,000 $\times$ g, 15 min) was used for the enzyme assay.

Thiamine kinase, thiamine monophosphate kinase, and thiamine pyrophosphokinase were assayed by measuring the reaction product of thiamine monophosphate or thiamine pyrophosphate directly by HPLC by the method of Shigeoka *et al.*³⁾ The reaction mixture (1 ml) of thiamine

kinase and thiamine pyrophosphokinase contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM MgCl₂, 1 mM KCl, 5 mM ATP, 25 μ M thiamine and enzyme. The reaction for 30 min at 30°C was started by adding thiamine and stopped by boiling for 1 min. After centrifuging at 10,000 $\times$ g for 3 min to remove denaturated protein, the reaction product in the supernatant was measured by HPLC. The assay for thiamine monophosphate kinase was like that for thiamine kinase except that thiamine monophosphate was the substrate. The specific activity of the kinases was expressed as pmol of thiamine phosphate ester formed/hr/mg protein. Protein was measured by the method of Bradford⁶⁾ with bovine serum albumin as a standard protein.

A cell homogenate of *E. gracilis* was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption and fractionated by differential centrifugation as described previously.⁷⁾ Ribulosebiphosphate carboxylase,⁸⁾ a chloroplast marker enzyme, succinate semialdehyde dehydrogenase,⁹⁾ a mitochondrial marker enzyme, glucose-6-phosphatase,¹⁰⁾ a microsomal marker enzyme and glutamate dehydrogenase,¹¹⁾ a cytosolic marker enzyme, were assayed by the methods of the cited references.

HPLC analysis of the reaction mixture using thiamine as a substrate showed the reaction product to be eluted with the same retention time of 6.7 min as that of authentic thiamine pyrophosphate, but not by thiamine monophosphate. The activity of the enzyme which catalyzes the direct conversion of thiamine to thiamine pyrophosphate was 376.7 pmol/hr/mg protein in the crude extract of *E. gracilis*, but neither thiamine kinase nor thiamine monophosphate kinase activity was detected. These results show that thiamine pyrophosphokinase occurs in *E. gracilis* and that thiamine is enzymatically converted to thiamine pyrophosphate.

Table I shows the contents of thiamine and its phosphate esters and the activities of thiamine pyrophosphokinase and marker enzymes in the subcellular fractions from differential centrifugation of a homogenate of *E. gracilis*. The activities of marker enzymes for chloroplasts, mitochondria, and microsomes were high in each compartment and low in the cytosol. Of thiamine, 12.1% was in the chloroplasts, 25.4% in mitochondria, and 60.6% in cytosol, with the content of crude homogenate as 100%, as judged from leakage of marker enzymes. Cytosol had 60.4% of the total thiamine pyrophosphate, mitochondria 35.2%, and chloroplasts 4.3%. Thiamine pyrophosphokinase was distributed 9.2% in chloroplasts, 15.7% in mitochondria, and 65.7% in cytosol. Sonication, treatment with Triton X-100, and then osmotic treatment of the mitochondrial and chloroplast fractions readily released the kinase as well as succinate semialdehyde dehydrogenase, a mitochondrial matrix enzyme, and ribulosebiphosphate carboxylase, a chloroplast stroma enzyme, indicating that the kinases in mitochondria and chloroplasts are soluble enzymes.

TABLE I. DISTRIBUTION OF THIAMINE, THIAMINE PHOSPHATE ESTERS, THIAMINE PYROPHOSPHOKINASE AND MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF THIAMINE-SUFFICIENT *Euglena* CELLS

Crude homogenate contained 149.1 nmol of thiamine, 1.2 nmol of thiamine monophosphate and 16.2 nmol of thiamine pyrophosphate. Distribution of thiamine, thiamine esters, and thiamine pyrophosphokinase in organelles is given as the percent distribution, after correction for leakages of marker enzymes.

	Percent of contents of thiamine and its phosphate esters and enzyme activities in crude homogenate			
	Chloroplasts	Mitochondria	Microsomes	Cytosol
Thiamine	12.1	25.4	1.4	60.6
Thiamine monophosphate	Trace	33.3	0	Trace
Thiamine pyrophosphate	4.3	35.2	1.2	60.4
Thiamine pyrophosphokinase	9.2 (34)	15.7 (284)	0	65.7 (186)
Ribulosebiphosphate carboxylase	89.7	0	0	4.5
Succinate semialdehyde dehydrogenase	3.1	97.7	0	0
Glucose-6-phosphatase	0	0	93.1	5.6
Glutamate dehydrogenase	0	0	3.2	98.2

* Specific activity (pmol/hr/mg protein) given in parenthesis.

The specific activity of the thiamine pyrophosphokinase of chloroplasts was much lower than that of the mitochondrial one in *Euglena gracilis* (Table I). The chloroplast enzyme was so labile that it was completely inactivated for 5 hr at 4°C, but the mitochondrial and cytosolic enzymes retained full activities up to 30°C between pH 6.0 and 9.0 and up to 40°C for 20 min between pH 8.0 and 9.0, respectively. The optimum pH and temperature of the mitochondrial kinase were 8.0 and 35°C, respectively, while those of the cytosolic one 7.0 and 50°C, respectively. In the mitochondrial enzyme, the apparent *K_m* value for thiamine was 0.63 µM and 57.5 µM for ATP, while in the cytosolic enzyme, the *K_m* values for thiamine and ATP were 0.91 µM and 48.5 µM, respectively.

These results demonstrate for the first time in *E. gracilis*, a eukaryote, that thiamine pyrophosphokinases exist as three different types in chloroplasts, mitochondria, and cytosol, suggesting the occurrence of isozymes. Thiamine pyrophosphokinase has been found in some organisms: yeast,⁽¹⁾ *Paracoccus denitrificans*,⁽²⁾ liver⁽³⁾ and brain⁽⁴⁾ in rats, heart⁽⁵⁾ and brain⁽⁶⁾ in pigs, and parsley leaves.⁽⁷⁾ However, no information was available on the occurrence of thiamine pyrophosphokinase isozymes. Furthermore, it is suggested that thiamine fed exogenously is incorporated into cytosol and further into each organelle and transformed to coenzyme by the respective thiamine pyrophosphokinase isozymes operating for thiamine pyrophosphate-dependent 2-oxoglutarate decarboxylase⁽⁸⁾ and pyruvate dehydrogenase⁽⁹⁾ in *Euglena* mitochondria, acetolactate synthase⁽²⁰⁾ in mitochondria and cytosol, transketolase in chloroplasts and cytosol (unpublished data), and several other enzymes. The mechanism of incorporation of thiamine into each organelle will be the subject of our

forthcoming paper.

REFERENCES

- 1) Y. Kaziro, R. Tanaka, Y. Mano and N. Shimazono, *J. Biochem.*, **49**, 472 (1961).
- 2) H. Nakayama and R. Hayashi, *J. Bacteriol.*, **109**, 936 (1972).
- 3) S. Shigeoka, T. Onishi, Y. Nakano and S. Kitaoka, *J. Gen. Microbiol.*, **133**, 25 (1987).
- 4) S. Shigeoka, T. Onishi, K. Maeda, Y. Nakano and S. Kitaoka, *Biochim. Biophys. Acta*, **929**, 247 (1987).
- 5) L. E. Koren and S. H. Hutner, *J. Protozool.*, **14**, Suppl. 17 (1967).
- 6) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1979).
- 7) S. Shigeoka, R. Yasumoto, T. Onishi, Y. Nakano and S. Kitaoka, *J. Gen. Microbiol.*, **133**, 227 (1987).
- 8) H. Rabinowitz, A. Resifeld, D. Sagher and M. Edelman, *Plant. Physiol.*, **56**, 345 (1975).
- 9) M. Tokunaga, Y. Nakano and S. Kitaoka, *Biochim. Biophys. Acta*, **429**, 55 (1976).
- 10) C. De Duve, B. C. Pressman, R. Gianetto, T. G. Wattiaux and F. Appelmans, *Biochem. J.*, **60**, 604 (1955).
- 11) M. Tokunaga, Y. Nakano and S. Kitaoka, *J. Protozool.*, **26**, 471 (1979).
- 12) H. Sanemori, T. Kawasaki, *J. Biochem.*, **88**, 223 (1980).
- 13) Y. Mano, *J. Biochem.*, **47**, 24 (1960).
- 14) L. R. Johnson and C. J. Gubber, *Biochim. Biophys. Acta*, **156**, 85 (1968).
- 15) Z. Suzuki, K. Furuno, K. Murakami, T. Fujita, T. Matsuoka and K. Takeda, *J. Nutr.*, **94**, 426 (1968).

- 16) J. W. Peterson, C. J. Gubler and S. A. Kuby, *Biochim. Biophys. Acta*, **397**, 377 (1975).
 - 17) H. Mitsuda, Y. Takii, K. Iwami and K. Yasumoto, *J. Nutr. Sci. Vitaminol.*, **21**, 103 (1975).
 - 18) S. Shigeoka, T. Onishi, K. Maeda, Y. Nakano and S. Kitaoka, *FEBS Lett.*, **195**, 43 (1986).
 - 19) H. Inui, K. Miyatake, Y. Nakano and S. Kitaoka, *J. Biochem.*, **96**, 931 (1984).
 - 20) Y. Oda, Y. Nakano and S. Kitaoka, *J. Gen. Microbiol.*, **128**, 1211 (1982).
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