The effect of thiamin on the activation of thiamin pyrophosphate-dependent 2-oxoglutarate decarboxylase in *Euglena gracilis*

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The effect of thiamin on thiamin pyrophosphate-dependent 2-oxoglutarate (2-OG) decarboxylase activity in *Euglena gracilis* was investigated. The total activity of 2-OG decarboxylase in thiamin-sufficient cells is 3 times that in thiamin-deficient cells. The addition of thiamin to thiamin-deficient cells causes the total enzyme and holoenzyme activities to increase and reach similar levels to that in thiamin-sufficient cells. Cycloheximide and chloramphenicol, inhibitors of protein synthesis, have no effect on the total enzyme activity. Immunochemical titration and determination of 2-OG decarboxylase mRNA by using an antibody directed against *Euglena* 2-OG decarboxylase indicate

that the increase in the holoenzyme activity of 2-OG decarboxylase is due to activation of pre-existing protein and does not require synthesis of new proteins in thiamin-deficient cells. During the period of the increase in the total activity, the apoenzyme increases and reaches a temporary peak in 2 h. Immunoblot analysis demonstrates that the precursor form (a 65 kDa subunit) of 2-OG decarboxylase in thiamin-deficient cells is more abundant than that in thiamin-sufficient cells and the increase in the apoenzyme by addition of thiamin results from the conversion of the precursor form into the mature form (a 62 kDa subunit).

INTRODUCTION

Euglena gracilis absolutely requires thiamin for growth, due to the lack of a biosynthetic pathway for the formation of the pyrimidine moiety [1]. Thiamin exhaustion in a medium allows *Euglena* cells (thiamin-sufficient cells) to suppress cell division and to decrease significantly the cellular content of thiamin and the phosphate esters. The addition of thiamin to thiamin-deficient cells causes the cells to return to the normal state [1].

The *Euglena* thiamin pyrophosphate (TPP)-dependent decarboxylation of 2-oxo acids, pyruvate and 2-oxoglutarate (2-OG), is unique [2]. *Euglena* mitochondria lack the pyruvate dehydrogenase complex and the 2-OG dehydrogenase complex of other living organisms, and instead contain novel pyruvate dehydrogenase and 2-OG decarboxylase. Pyruvate dehydrogenase is an oxygen-sensitive and NADP⁺-dependent enzyme and plays a physiologically important role in wax ester fermentation [3]. 2-OG decarboxylase, which catalyses the conversion of 2-OG into succinate semialdehyde, is one of the constituent enzymes together with succinate semialdehyde dehydrogenase in the *Euglena* tricarboxylic acid cycle [4].

We have recently purified and characterized 2-OG decarboxylase from thiamin-sufficient cells [5]. 2-OG decarboxylase requires TPP and MgCl₂ as cofactors. NADP⁺ and its analogues increase significantly the enzyme activity. In addition, as L-glutamate added exogenously accumulates in *Euglena* cells, 2-OG decarboxylase is induced to assimilate the amino acid [6]. On the basis of these results and other enzymological and molecular properties, we suggested that 2-OG decarboxylase is an important regulatory enzyme in the tricarboxylic acid cycle of *Euglena* mitochondria. Furthermore, 2-OG decarboxylase has a molecular mass of 250 kDa, consisting of four identical subunits of molecular mass 62 kDa. Translation *in vitro* of polyadenylated RNA and immunoblot analysis using an antibody directed against 2-OG decarboxylase indicates that a putative precursor

of 2-OG decarboxylase which is about 3 kDa larger than the subunit of the mature enzyme is synthesized.

In this context, we were interested in the effect of thiamin on TPP-dependent 2-OG decarboxylase activity in *Euglena* cells, in order to substantiate in detail the regulatory system of the enzyme in the tricarboxylic acid cycle. First we investigated the effect of thiamin on the 2-OG decarboxylase activity in thiamin-deficient cells. In addition, the molecular mechanism of the increase in 2-OG decarboxylase activity caused by addition of thiamin to the thiamin-deficient cells was studied by using an antibody directed against *Euglena* 2-OG decarboxylase.

EXPERIMENTAL

Materials

The antibody raised against *Euglena* 2-OG decarboxylase was prepared as described previously [5]. L-[³⁵S]Methionine (1056 Ci/mmol) and rabbit reticulocyte lysate were obtained from Amersham, Tokyo, Japan. Thiamin, TPP, oligo(dT)-cellulose and Protein A-Sepharose were purchased from Sigma Chemical Co. All reagents for SDS/PAGE and goat anti-IgG serum-peroxidase conjugate were from Pharmacia Fine Chemicals. All other chemicals were of reagent grade.

Culture and organism

E. gracilis Z, thiamin-sufficient cells, were cultured in 50 ml of Koren–Hutner medium [7] in the presence of 35 µg of thiamin for 5 days under illumination (3000 lx) at 26 °C. Thiamin-deficient cells were prepared as described by Shigeoka et al. [1]. Thiamin-sufficient cells (13 × 10⁶ cells) were transferred to basal medium (150 ml) in the absence of thiamin and cultured for 5 days to obtain thiamin-limited cells (5 × 10⁶ cells/ml). Cultivation for thiamin-deficient cells (10⁶ cells/ml) was performed in the same manner as described above, but with 1 ml of thiamin-

limited cells. Thiamin-supplemented cells were obtained by adding 60 nM thiamin to thiamin-deficient cells in stationary phase [1]. Cell number was counted with a haemocytometer.

Preparation of crude extract

Euglena cells were harvested by centrifugation, washed twice, resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 20% (w/v) ethylene glycol and disintegrated by sonication (10 kHz) for 2 min. The lysate was centrifuged at 10000 *g* for 10 min, and the supernatant was used as a crude extract.

Enzyme assay

2-OG decarboxylase was assayed by the colorimetric *o*-aminobenzaldehyde method as described previously [4]. The total reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 5 mM 2-OG, 1 mM MgCl₂, 0.2 mM TPP, 1 mM NADP⁺, 1 mM 2-mercaptoethanol and enzyme in a total volume of 2 ml. This represents the total activity of 2-OG decarboxylase. The reaction mixture in which TPP and MgCl₂ were omitted from the total assay was used to determine the activity of holoenzyme. The difference of enzyme activities between the total assay and the holoenzyme assay represents the apoenzyme of 2-OG decarboxylase.

Immunoblot analysis

Western immunoblotting using an antibody directed against *Euglena* 2-OG decarboxylase was carried out by the method described previously [5]. The crude extract was subjected to SDS/PAGE on 7.5–10%-polyacrylamide slab gels. Proteins were transferred electrophoretically to a nitrocellulose membrane by using a semi-dry blotting apparatus (Bio-Rad). Immunodetection of proteins on blots was done at room temperature with 0.5% (w/v) skim milk in 20 mM Tris/HCl buffer, pH 8.3, containing 100 mM NaCl as a blocking agent. Peroxidase-conjugated goat anti-(rabbit IgG) serum was used as a secondary antibody.

Immunochemical titration

This was performed as described by Shigeoka et al. [6]. Crude extracts were prepared as described above from thiamin-sufficient cells and from thiamin-supplemented cells, 2 h after the addition of thiamin. Different amounts of both crude extracts were incubated with 5 μ l of anti-(2-OG decarboxylase) antibody (615 μ g of protein) for 60 min at 30 °C. The insoluble complexes were removed by centrifugation at 10000 *g* for 15 min, and the enzyme activity remaining in the supernatant was determined.

Extraction and translation *in vitro* of polyadenylated RNA

Extraction of total RNA and isolation of polyadenylated RNA (mRNA) by using oligo(dT)-cellulose from thiamin-deficient and -supplemented *Euglena* cells were performed as described previously [5]. A 25 μ l rabbit reticulocyte cell-free translation system was charged with 10 μ Ci of L-[³⁵S]methionine as radioactive label [8] and 0.3 μ g of polyadenylated mRNA, this being the optimal concentration for programming protein synthesis

Figure 1 Effect of thiamin and cycloheximide on 2-OG decarboxylase activity

(a) Thiamin (60 nM) was added to thiamin-deficient cells grown for 5 days. (b) Cycloheximide was added at a final concentration of 0.1 mM, concomitantly with thiamin, which did not affect the cell viability [6,10]. Total (○) and holoenzyme (●) activities of 2-OG decarboxylase were assayed by the colorimetric *o*-aminobenzaldehyde method (see the Experimental section). Each point represents the mean of three assays (coefficient of variation < 5%).

[5,6]. The products of translation of total mRNA were obtained as trichloroacetic acid precipitates. 2-OG decarboxylase proteins translated *in vitro* were immunoprecipitated with a specific antibody and Protein A-Sepharose. Radioactivity in total proteins and 2-OG decarboxylase was measured by a scintillation photometer (model LSC-903; Aloka, Tokyo, Japan).

Protein assay

Protein was determined by the method of Bradford [9] with BSA as a standard.

RESULTS

Effect of thiamin on 2-OG decarboxylase activity

Thiamin-sufficient cells grown for 5 days contained total 2-OG decarboxylase activity of 3.61 ± 0.08 nmol/min per 10^6 cells; the holoenzyme activity accounted for about 50% of the total enzyme activity. In thiamin-deficient cells in stationary phase, the total enzyme activity was 1.25 ± 0.06 nmol/min per 10^6 cells, but the apoenzyme comprised 100% of the total activity. When 60 nM thiamin was added to thiamin-deficient cells, the total activity of 2-OG decarboxylase increased about 3-fold over 2 h (Figure 1a). A similar increase in the holoenzyme activity was also observed; the holoenzyme activity comprised about 50% of the total activity over 12 h. The simultaneous addition of cycloheximide (0.1 mM), an inhibitor of protein synthesis on 87S cytoplasmic ribosomes [6,10], had no effect on the increase in the enzyme activity and the ratio of the holoenzyme/total enzyme activity (Figure 1b). Chloramphenicol (3 mM), an inhibitor of protein synthesis on 68S plastid ribosomes [10], also did not inhibit the increase.

Effect of thiamin on the synthesis of 2-OG decarboxylase protein

In order to confirm whether the increase in the total 2-OG decarboxylase activity is caused by the biosynthesis of the enzyme

Figure 2 Immunochemical titration of 2-OG decarboxylase from thiamin-deficient cells (0 h; t_0) and -supplemented cells (2 h; t_2)

Crude extracts of t_0 (●) and t_2 (○) samples were prepared, and immunochemical titration with anti-(2-OG decarboxylase) antibody was carried out as described in the Experimental section. The abscissa expresses the 2-OG decarboxylase activity before immunochemical reaction and the ordinate expresses the activity remaining in the 10000 g supernatant after removal of any antigen-antibody complex. Each experimental point represents the mean of three assays (coefficient of variation < 7%).

Figure 3 Effect of thiamin on 2-OG decarboxylase mRNA and total mRNA activities

Total RNA was extracted from thiamin-deficient cells (t_0) and thiamin-supplemented cells (t_1 , t_2 and t_4) and polyadenylated RNA was then isolated. The polyadenylated RNA (0.3 μ g) was used for translation *in vitro* in rabbit reticulocyte lysates in the presence of L-[35 S]methionine. The products from polyadenylated RNA (●) were obtained as trichloroacetic acid precipitates. 2-OG decarboxylase mRNA (○) was determined by immunoprecipitation of the enzyme protein with a specific antibody. Each point represents the mean of three assays (coefficient of variation < 6%).

or the activation of pre-existing protein, immunochemical titration was performed on the cell-free extracts prepared from thiamin-deficient cells at t_0 (0 h) and thiamin-supplemented cells at t_2 (2 h). When the activity of 2-OG decarboxylase at t_0 and t_2 was inhibited by a fixed amount of anti-(2-OG decarboxylase) antibody, the amount of 2-OG decarboxylase activity was unequal for both enzyme preparations (Figure 2). Therefore, although there is no enzyme activity in thiamin-deficient cells, the protein is present and the observed increase in 2-OG decarboxylase activity over the time t_0 – t_2 is due to the activation of pre-existing protein, but not to synthesis *de novo* of the 2-OG decarboxylase protein.

Table 1 Effect of compounds on the increase in 2-OG decarboxylase activity

Each compound was added to thiamin-deficient cells to the final concentration shown, which did not affect cell viability, 1 h before addition of thiamin. The effect is represented as percentage inhibition against the increase in total 2-OG decarboxylase activity in the respective control to which no compound was added. Values are means of three assays $\pm$ S.D.

Compounds	Concn.	Enzyme activity (nmol/min per 10^6 cells)	Inhibition (%)
None			
0 h		1.36 $\pm$ 0.15	
1 h		3.26 $\pm$ 0.21	0
Cycloheximide	(50 μ M)	3.23 $\pm$ 0.25	2
Chloramphenicol	(0.1 mM)	3.26 $\pm$ 0.24	0
1,10-Phenanthroline	(3 mM)	2.55 $\pm$ 0.19	37
2,4-Dinitrophenol	(2 μ M)	2.48 $\pm$ 0.21	41
CCCP	(1 mM)	2.12 $\pm$ 0.12	60

Figure 4 Effect of thiamin on conversion of the precursor of 2-OG decarboxylase into the mature form

The crude extracts from thiamin-deficient cells (t_0) and thiamin-supplemented cells (t_1 , t_2 and t_4) were subjected to SDS/PAGE in a 7.5–10% linear gradient polyacrylamide slab gel and transferred to a nitrocellulose membrane by Western blotting as described previously [5]. Proteins were detected immunologically by using an antibody directed against 2-OG decarboxylase and peroxidase-conjugated goat anti-(rabbit IgG) serum. Lanes: 1, t_0 ; 2, t_1 ; 3, t_2 ; 4, t_4 .

Effect of thiamin on 2-OG decarboxylase mRNA content

Polyadenylated RNA was extracted from thiamin-deficient cells and from thiamin-supplemented cells at t_1 (1 h), t_2 and t_4 (4 h). 2-OG decarboxylase mRNA was measured by immunoprecipitation of products of translation *in vitro* of mRNA in lysates using an anti-(2-OG decarboxylase) antibody. Translatability of mRNA (0.3 μ g) after addition of thiamin was unaltered, with each time point giving similar incorporations per lysate (results not shown). Radioactivity obtained for products of translation *in vitro*, presented per 10^6 cells, increased linearly up to 4 h after the supplement of thiamin. However, there was no apparent difference in the amount of 2-OG decarboxylase mRNA between thiamin-deficient cells (t_0) and thiamin-supplemented cells (t_1 , t_2 and t_4) (Figure 3).

Effect of compounds on 2-OG decarboxylase activity

Table 1 shows the effect of some compounds added to thiamin-deficient cells together with the addition of thiamin on the increase in total 2-OG decarboxylase activity. 1,10-Phenanthroline, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazide (CCCP) considerably inhibited the increase in 2-OG decarboxylase activity.

Figure 5 Effect of some compounds on conversion of the precursor of 2-OG decarboxylase into the mature form

Crude extracts from thiamin-deficient cells (t_0) and thiamin-supplemented cells (t_1) after the simultaneous addition of each compound were prepared. Western-blot analysis and the immunological detection of proteins were performed in accordance with the method in Figure 4. Lanes: 1, t_0 ; 2, $t_1 + 2.5$ mM 1,10-phenanthroline; 4, $t_1 + 2$ μ M 2,4-dinitrophenol; 5, $t_1 + 1$ mM chloramphenicol; 6, $t_1 + 1$ mM CCCP.

Effect of thiamin on conversion of the precursor form into the mature form of 2-OG decarboxylase

Thiamin-sufficient cells contain two polypeptides, with molecular masses of 62 kDa and 65 kDa, which cross-reacted with an antibody directed against 2-OG decarboxylase. The 62 kDa mature form comprises about 80 % of total 2-OG decarboxylase protein [5]. On the basis of immunoblot analysis, the precursor protein of the enzyme existed predominantly, in comparison with the mature enzyme, in the crude extract from thiamin-deficient cells (Figure 4). Once thiamin was supplied to thiamin-deficient cells, the transfer from the premature form to the mature form of 2-OG decarboxylase was observed over 2 h in thiamin-supplemented cells. As shown in Figure 5, the concomitant addition of 1,10-phenanthroline, 2,4-dinitrophenol or CCCP together with thiamin markedly inhibited the change of the enzyme form, whereas chloramphenicol had no effect.

DISCUSSION

Thiamin is absolutely required for optimal growth of *Euglena*. Thus, the depletion of thiamin causes the arrest of cell division. Thiamin-deficient cells contain one-tenth of the amount of thiamin, and only traces of thiamin monophosphate and TPP, in comparison with those in thiamin-sufficient cells [1]. When 60 nM thiamin was added to thiamin-deficient cells grown for 5 days in the absence of thiamin, cell division commenced and the cell number reached the same level as with thiamin-sufficient cells within 2 days. The total 2-OG decarboxylase activity in thiamin-deficient cells decreased to 30 % of that in thiamin-sufficient cells; the activity of holoenzyme was not found in thiamin-deficient cells. Supplementation of 60 nM thiamin to thiamin-deficient cells caused the total enzyme and holoenzyme activities to increase immediately without a lag phase and to reach a peak in 2 h (Figure 1). The addition of cycloheximide together with thiamin had no effect on the increase in activities of total enzyme and holoenzyme. The immunochemical titration using anti-(2-OG decarboxylase) antibody was performed on extracts of thiamin-deficient and -supplemented cells. The titration experiment showed that the same amount of 2-OG decarboxylase at t_0 and t_2 was not inhibited by a fixed amount of antibody (Figure 2). The content of 2-OG decarboxylase mRNA did not change after the supplement of thiamin, whereas that of total mRNA increased linearly over 4 h (Figure 3). From these results reported here, we interpreted that the observed increase in the holoenzyme activity of 2-OG decarboxylase results from the activation of the pre-

existing protein in the cells, but not from a corresponding increase in the amount of enzyme protein.

When 60 nM thiamin was added to thiamin-deficient cells, it was taken up for 2 h by a carrier-mediated active transport process [11]. We have previously demonstrated that thiamin pyrophosphokinase, which catalyses the conversion of thiamin into thiamin pyrophosphate, exists as three different types in chloroplasts, mitochondria and cytosol [12]. Therefore, it is conceivable that thiamin is taken up and converted into TPP by pyrophosphokinase, and then TPP acts as a coenzyme converting holoenzyme from the apoenzyme of 2-OG decarboxylase pre-existing in thiamin-deficient cells. However, the apoenzyme temporarily increased after addition of thiamin to thiamin-deficient cells (Figure 1). This result does not sufficiently account for the activation of the total enzyme activity by addition of thiamin. There may be an inactive form of 2-OG decarboxylase in thiamin-deficient cells that is not represented when TPP is added to the reaction mixture. It has been reported that vitamin B₆-deficient rats contain an inactive apo-form of aspartate aminotransferase in liver and heart, and supplementation of pyridoxine causes the change from the inactive form into the active form [13].

How does the increase in apoenzyme occur in thiamin-supplemented cells? Analysis of the product of translation of *Euglena* total mRNA *in vitro* by reaction with anti-(2-OG decarboxylase) antibody and SDS/PAGE immunoprecipitated one product with a molecular mass of 65 kDa [5]. On immunoblot analysis of the crude extract of thiamin-sufficient cells, the antibody recognized two polypeptides with molecular masses of 62 and 65 kDa; the former was predominantly present, in comparison with the latter. The polypeptide with a molecular mass of 62 kDa was found only in the mitochondria fraction [5]. Therefore, 2-OG decarboxylase is synthesized initially as a precursor molecule, and is subsequently imported from the cytosol into mitochondria in its mature form [5]. We have recently demonstrated that when L-glutamate is added to *Euglena* cells grown on glucose/(NH₄)₂SO₄ medium, the 2-OG decarboxylase activity increases and reaches a peak after 4 h [6]. In addition, the effect of cycloheximide, immunochemical titration and labelling *in vivo* showed that the increase in 2-OG decarboxylase activity is due to the synthesis of new protein. Actually, the incorporation of L-[³⁵S]methionine into 2-OG decarboxylase protein was similar to the increase in 2-OG decarboxylase activity. Furthermore, on the basis of immunoblot analysis of the crude homogenate from *Euglena*, the precursor and mature enzyme proteins increase without altering the ratio of mature/precursor protein [6]. In the crude homogenate of thiamin-deficient cells, the precursor protein is more abundant than the mature protein, as distinct from that of thiamin-sufficient cells (Figure 4). When thiamin was added to thiamin-deficient cells, the change of 2-OG decarboxylase from the precursor form to the mature form was observed over 2 h. As shown in Table 1 and Figure 5, proteinase inhibitors and uncouplers considerably inhibited the increase in the enzyme activity and the conversion of the precursor into the mature form. These results reveal that the temporary increase in the apoenzyme is attributable to the change from the precursor form to the mature form.

When thiamin was added to thiamin-deficient cells, 96 % of total thiamin taken up was accumulated as thiamin, and the remainder was as phosphate esters [1]. Both the addition of 1 mM thiamin to the reaction mixture for the total enzyme assay and the incubation of 1 mM thiamin for 10 min with the crude extract from thiamin-deficient cells did not result in activation of the total 2-OG decarboxylase activity in thiamin-deficient cells

(results not shown). These results suggest that thiamin added exogenously is incorporated into cytosol and directly involved in the import of the precursor protein of 2-OG decarboxylase from cytosol to mitochondria. In summary, the activation of 2-OG decarboxylase by thiamin is caused by two mechanisms: the change from apoenzyme present in thiamin-deficient cells into holoenzyme by TPP produced during uptake of thiamin, and the change from the premature form into the mature form during the import into the mitochondria. In the control of enzyme activity studied so far, four regulatory motifs are observed in many enzymes; (1) allosteric interactions, such as haemoglobin and aspartate transcarbamoylase; (2) reversible covalent modification, such as phosphorylation of glycogen phosphorylase and adenylation of glutamine synthetase; (3) stimulation and inhibition by control proteins, such as calmodulin and anti-haemophilic factor; (4) proteolytic activation, such as chymotrypsin [14]. However, the increase in the apoenzyme of 2-OG decarboxylase by thiamin added exogenously is attributable to the conversion from the premature form to the mature form of 2-OG decarboxylase during import into the mitochondria from cytosol. In contrast, Inui et al. [15] have reported that thiamin-deficient cells lack pyruvate dehydrogenase protein and that addition of thiamin causes synthesis *de novo* of the enzyme. In addition, the new synthesized protein existed totally as holoenzyme. These results mean that the cellular thiamin and/or TPP regulate the activation and synthesis of TPP-dependent enzymes in *Euglena*. Detailed studies on the involvement of thiamin in the importation of the precursor enzyme into mitochondria remain to be done.

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