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Occurrence of thiamin pyrophosphate-dependent 2-oxoglutarate decarboxylase in mitochondria of *Euglena gracilis*

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2-Oxoglutarate decarboxylase which catalyzes the conversion of 2-oxoglutarate into succinate semialdehyde occurs in mitochondria of *Euglena gracilis* which lacks a 2-oxoglutarate dehydrogenase complex. The enzyme reaction required thiamin pyrophosphate, $MgCl_2$, 2-mercaptoethanol and $NADP^+$ for the maximum activity, and was not affected by pyruvate and oxalacetate. In the reaction, the enzyme consumed 2-oxoglutarate, evolved CO_2 and formed succinate semialdehyde in stoichiometric relationship. The maximum enzyme activity was found at pH 7.0 and 40°C, and K_m values for 2-oxoglutarate and thiamin pyrophosphate were 0.33 and 0.056 mM, respectively. These results indicate that the thiamin pyrophosphate-dependent *Euglena* decarboxylase belongs to a new type of decarboxylase to be designated as 2-oxoglutarate decarboxylase. The probable role of the new decarboxylase in *Euglena* mitochondria is discussed with regard to the tricarboxylic acid cycle.

2-Oxoglutarate decarboxylase *Thiamin pyrophosphate* *Tricarboxylic acid cycle* *(*Euglena *mitochondria)*

1. INTRODUCTION

We have reported in a previous paper that *Euglena* shows an absolute growth requirement for vitamin B_1 [1]. In thiamin pyrophosphate-dependent decarboxylation of 2-oxo acid, *Euglena* contained an oxygen-sensitive and $NADP^+$-dependent pyruvate dehydrogenase that is distinct from the pyruvate dehydrogenase complex in other organisms, but which is rather similar to pyruvate synthase (EC 1.2.7.1) found in anaerobic organisms [2].

This paper reports that thiamin pyrophosphate-dependent 2-oxoglutarate decarboxylase occurs in *Euglena* mitochondria which lacks a 2-oxoglutarate dehydrogenase complex (EC 1.2.4.2 + EC 2.3.1.61 + EC 1.6.4.3) and that the enzyme has a physiologically important role coupling with succinate-semialdehyde dehydrogenase (EC 1.2.1.16) in the tricarboxylic acid cycle.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

o-Aminobenzaldehyde from *o*-nitrobenzaldehyde and succinate semialdehyde were prepared as in [3] and [4], respectively. 2-[U-^{14}C]Oxoglutaric acid (256 mCi/mmol) was purchased from New England Nuclear. All other chemicals were reagent grade obtained from commercial sources.

2.2. *Cell culture*

E. gracilis Z was cultured in Koren-Hutner medium [5] under illumination (3000 lux) with shaking at 26°C for 5 days.

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2.3. *Subcellular localization*

A homogenate of *Euglena* cells was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption and then submitted to differential centrifugation for subcellular fractionation [6]. Ribulose-bisphosphate carboxylase (EC 4.1.1.39) [7] was used as a marker enzyme of chloroplasts; succinate-semialdehyde dehydrogenase [8] as a mitochondrial marker enzyme; glucose-6-phosphatase (EC 3.1.3.9) [9] as a microsomal marker enzyme and glutamate dehydrogenase (EC 1.4.1.4) [10] as a cytosolic marker enzyme.

2.4. *Enzyme assays*

The activity of 2-oxoglutarate decarboxylase was assayed by measuring radioactivity in evolved CO_2 or the formed succinate semialdehyde. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 5 mM 2-oxoglutarate (0.05 μCi), 1 mM $MgCl_2$, 0.2 mM thiamin pyrophosphate, 1 mM $NADP^+$, 1 mM 2-mercaptoethanol and enzyme in a total volume of 2.0 ml. Incubation was carried out at 30°C for 10–30 min and the reaction stopped by the addition of 0.1 ml of 25% trichloroacetic acid. The succinate semialdehyde formed was determined colorimetrically by the *o*-aminobenzaldehyde method [11]. Labeled CO_2 evolved during incubation was trapped by 2 N KOH and the radioactivity was counted with a scintillation photometer (Aloka, model LSC-903).

2-Oxoglutarate dehydrogenase complex activity was assayed according to [12]. The 3 component enzymes, 2-oxoglutarate dehydrogenase (EC 1.2.4.2) [13], lipoate succinyltransferase (EC 2.3.1.61) [14] and lipoamide dehydrogenase (EC 1.6.4.3) [15], were assayed by the respective methods. Protein was determined as in [16].

2.5. *Identification of the reaction product*

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were employed for the identification of the reaction product. The enzyme reaction (30 min) was stopped by the addition of HCl and 2,4-dinitrophenylhydrazine. The mixture was incubated for 1 h at 50°C and the hydrazones formed were extracted with ethyl acetate. The extract was evaporated to dryness in vacuo and the hydrazones were dissolved with a small portion of ethyl acetate. The solution was applied onto a thin layer of Kieselguhr F_{254} and developed with *n*-butanol saturated with 3% ammonium hydroxide. In separation of reaction products by HPLC, the reaction mixture was applied directly onto a column (120 × 8 mm i.d.) packed with IEX-520 QAE (Toyo Soda) and the column was eluted with 1/30 M tartaric acid-NaH_2PO_4 (pH 3.0):acetonitrile (72.5:27.5) at a flow rate of 1.5 ml/min and 25°C. The column eluate was monitored at 210 nm with a UV detector (Jasco, Uvidec-100). Authentic 2-oxoglutarate and succinate semialdehyde had retention times of 12.3 and 5.5 min, respectively.

3. RESULTS AND DISCUSSION

3.1. *Occurrence of 2-oxoglutarate decarboxylase*

Activities of CoA-dependent 2-oxoglutarate dehydrogenase complex and the 3 component enzymes were detected neither in crude homogenate nor in each subcellular fraction. However, crude homogenate did support the decarboxylation of 2-[U-^{14}C]oxoglutarate which could be assayed by measuring evolution of CO_2. As shown in table 1, this enzyme reaction depended on thiamin pyrophosphate and $MgCl_2$, but not CoA; the activities without thiamin pyrophosphate and $MgCl_2$ were about 10 and 45% of the maximum activity, respectively.

3.2. *Separation of 2-oxoglutarate decarboxylase*

Euglena cells (wet basis 20 g) were washed with 50 mM phosphate buffer (pH 7.0), containing 30% (w/v) ethylene glycol, and disrupted in 40 ml of the same buffer by sonication (10 kHz, 5 min). The supernatant obtained by centrifugation of the sonicate at 10000 × *g* for 20 min was used as crude enzyme. It was centrifuged at 105000 × *g* for

Table 1

Cofactor requirements for 2-oxoglutarate decarboxylase activity

Condition	Relative activity (%)
Complete	100
– thiamin pyrophosphate	10
– $MgCl_2$	45
+ CoA	100

CoA was added at 1 mM to the complete system

60 min with a Hitachi RP 65, the supernatant applied onto a DEAE-cellulose column (2.5 × 50 cm) and the column eluted with a 0–0.4 M linear gradient concentration of KCl. The elution pattern showed only one peak of 2-oxoglutarate decarboxylase activity with 0.25 M KCl. The enzyme preparation obtained had been partially purified 6.8-fold over the crude enzyme in 65.4% recovery.

3.3. *Identification of reaction product*

In TLC, a yellow spot of the hydrazone of the reaction product emerged with an R_f value of 0.31, indicating that it is identical with that of authentic succinate semialdehyde, different from the R_f value of 0.08 displayed by 2-oxoglutarate. Of the total radioactivity of 2-[U-^{14}C]oxoglutarate as a substrate, more than 40% was found in the succinate semialdehyde fraction. HPLC analysis of the reaction mixture showed the reaction product to be eluted with the same retention time of 5.3 min as shown by authentic succinate semialdehyde. These results show that succinate semialdehyde is the product of the enzyme reaction.

Table 2 lists the stoichiometry of 2-oxoglutarate consumption, succinate semialdehyde formation and CO_2 evolution. The results obtained agree fully with the predicted stoichiometry of the reaction.

3.4. *Some properties of 2-oxoglutarate decarboxylase*

The maximum activity was maintained up to 60°C between pH 6.0 and 8.7, but at 70°C the enzyme activity was almost completely lost. The enzyme reaction was optimum at pH 7.0 and 35–40°C. Mn^{2+}, Co^{2+} and Ca^{2+}, when added to the reaction mixture at 1 mM in place of Mg^{2+}, could support enzyme activity; however Hg^{2+}, Ni^{2+} and Cu^{2+} markedly inhibited the reaction; the effect was highest with Hg^{2+}.

Sulfhydryl compounds like 2-mercaptoethanol and dithiothreitol stimulated the enzyme activity by about 1.6-fold, while sulfhydryl inhibitors such as *N*-ethylmaleimide and *p*-chloromercuribenzoate were potent inhibitors, suggesting that a sulfhydryl group is concerned with the active center of the enzyme protein. $NADP^+$ (0.1 or 1 mM) augmented significantly the enzyme activity without change in the absorbance at 340 nm, while nicotinic acid, ATP, ADP and AMP had no significant effect on the activity. The mechanism of the enhancement of enzyme activity by $NADP^+$ is not clear at present.

In the presence of 1 or 5 mM oxalacetate in the reaction mixture together with 1 mM 2-oxoglutarate, the 2-oxoglutarate decarboxylase activity was about 93% of that evident in the absence of oxalacetate. Pyruvate had no effect on the activity. Titrations of enzyme activity with 2-oxoglutarate over the range 0–2.0 mM yielded a hyperbolic curve; however, above 2 mM inhibition was observed. The apparent K_m of the decarboxylase for 2-oxoglutarate was 0.33 mM. The enzyme obeyed the Michaelis-Menten equation over a wide range of thiamin pyrophosphate concentrations; the K_m for thiamin pyrophosphate was 0.056 mM. The results show that this enzyme has a high affinity toward 2-oxoglutarate and thiamin pyrophosphate. Thiamin pyrophosphate-dependent pyruvate decarboxylase (EC 4.1.1.1) [17] has a relatively low substrate specificity and acts weakly on 2-oxoglutarate, but no carboxylase is known which requires specifically 2-oxoglutarate as the substrate.

Table 2

Stoichiometry of 2-oxoglutarate decarboxylase

Reaction time (min)	2-Oxoglutarate consumed	CO_2 evolved	Succinate semialdehyde formed
10	1.54	1.46	1.56
30	4.65	4.66	4.70

Values expressed as μmol/mg protein

3.5. *Subcellular location of 2-oxoglutarate decarboxylase*

Table 3 shows the subcellular distribution of the activity of 2-oxoglutarate decarboxylase together with those of some marker enzymes. 2-Oxoglutarate decarboxylase was localized only in the mitochondria; it was absolutely not detected in microsomes and cytosol. The low activity (2%) in chloroplasts was due to contamination by the mitochondrial fraction, as judged from the distribution of succinate-semialdehyde dehydrogenase, a mitochondrial matrix enzyme. These results indicate that 2-oxoglutarate decarboxylase found on-

Table 3

Distribution of 2-oxoglutarate decarboxylase and marker enzymes in subcellular fractions of *Euglena*

Enzyme	Enzyme activity (% of activity in crude extract)			
	Chloroplasts	Mitochondria	Microsomes	Cytosol
2-Oxoglutarate decarboxylase	2.4	98.8	0	0
Ribulose-bisphosphate carboxylase	86.4	0	0	13.2
Succinate-semialdehyde dehydrogenase	4.0	102.0	0	0.1
Glucose-6-phosphatase	0	8.0	84.8	11.0
Glutamate dehydrogenase	0	0	1.7	94.8

ly in mitochondria of *Euglena* is obviously distinct from pyruvate decarboxylase located in cytosol, even if it is present in *Euglena* [18].

The present results show clearly that the thiamin pyrophosphate-dependent decarboxylase occurring in *Euglena* is distinct from any previously known decarboxylase in its subcellular location, the specific requirement of 2-oxoglutarate and other properties. It may be worth considering that *Euglena* decarboxylase should be classified as a new type of decarboxylase.

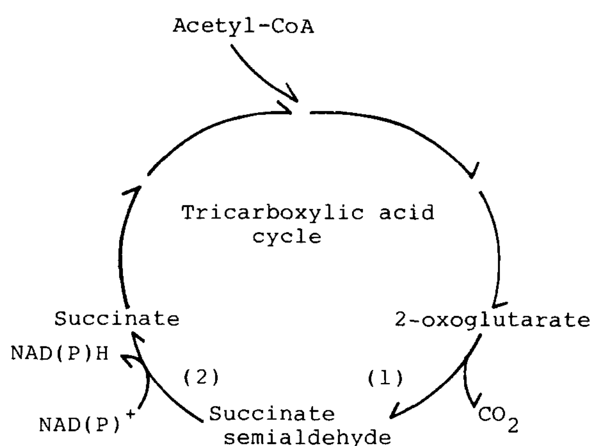

Fig.1. Outline of tricarboxylic acid cycle in *Euglena*. (1) 2-Oxoglutarate decarboxylase, (2) succinate-semialdehyde dehydrogenase.

3.6. *Probable physiological role of 2-oxoglutarate decarboxylase*

It has been reported that *Euglena* mitochondria contain 2 succinate-semialdehyde dehydrogenases (EC 1.2.1.16), one requiring NAD^+ and the other $NADP^+$, which catalyze the conversion of succinate semialdehyde to succinate. The $NADP^+$-linked isoenzyme has an approx. 8.5-times higher activity than the NAD^+-linked isoenzyme [8]. During the purification of 2-oxoglutarate decarboxylase reported here, the $NADP^+$-linked succinate-semialdehyde dehydrogenase was eluted at 0.025 M KCl with DEAE-cellulose column chromatography. In the presence of partially purified 2-oxoglutarate decarboxylase and succinate-semialdehyde dehydrogenase, 2-oxoglutarate as a substrate was converted stoichiometrically into succinate with concomitant formation of NADPH.

The facts reported here indicate that the eukaryotic cells of *Euglena*, which are devoid of the 2-oxoglutarate dehydrogenase complex, have an alternative route in the tricarboxylic acid cycle. This appears to be operated by a unique 2-oxoglutarate decarboxylase and succinate-semialdehyde dehydrogenase as shown in fig.1. Furthermore, the fact that succinate semialdehyde acts as the best respiratory substrate in intact *Euglena* mitochondria [19] supports the above physiological operation of a new route.

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