Wax ester fermentation in *Euglena gracilis*

Hiroshi Inui, Kazutaka Miyatake, Yoshihisa Nakano and Shozaburo Kitaoka*

*Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 591, Japan*

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Aerobically grown *Euglena gracilis*, a bleached mutant, shows a prompt synthesis of wax esters with the concomitant fall of the paramylon (a β-1,3-glucan) content upon exposure to anaerobiosis. Bringing the anaerobic cells back to aerobiosis causes the reverse conversions. The anaerobic wax ester formation is accompanied by a net synthesis of ATP. The transition between the fermentation and respiration occurs at $10^{-5}-10^{-7}$ M of the $O_2$ concentration.

**Fermentation Wax esters**

**Euglena gracilis**  
**Paramylon O$_2$ concentration**

1. INTRODUCTION

Wax esters are formed in *Euglena gracilis* when grown in an organic carbon-rich medium under adverse conditions [1-5]. We have found that transfer of aerobically grown *E. gracilis* to anaerobic conditions causes a prompt formation of wax esters at the expense of paramylon, the reserve polysaccharide. The anaerobic wax ester formation is accompanied by a net synthesis of ATP.

2. MATERIALS AND METHODS

2.1. Cells and cultures

*Euglena gracilis* SM-ZK, a streptomycin-bleached mutant [6] derived from strain Z, was used throughout experiments. Cells were cultured in a medium as in [7] under illumination (2000 lux) with shaking (90 strokes/min) at 27°C. The cells employed in chase label experiments, containing labelled paramylon, were obtained by culturing *Euglena* in the medium containing [U-14C]glucose (4 μCi/150 ml); of the incorporated radioactivity 75% was present in the paramylon fraction.

2.2. Anaerobiosis

Cells were harvested by centrifugation (1500 × g, 2 min) and suspended at 27°C in 2 ml potassium phosphate buffer (pH 6.8) to 2 × 10$^7$ cells/ml. Anaerobiosis was applied by bubbling N$_2$ which had passed through 15% potassium hydroxide solution containing 10% pyrogallol and then distilled water. O$_2$ in the suspension was $\sim10^{-8}$ M. For obtaining N$_2$–O$_2$ mixtures with different, precise O$_2$ concentrations, an oxygen pump (Toray, model SEP-104) was employed.

2.3. Analyses of cells components

Cells in the 2 ml suspension were collected by centrifugation (3000 × g, 2 min) and washed with distilled water, and added into 4.75 ml of a mixture of chloroform–methanol–water (10:20:8, by vol.). After thorough agitation the mixture was centrifuged to remove cells and debris. The extraction was repeated, and to the combined supernatants were added 2 ml each of chloroform and water, and the whole was shaken vigorously. The chloroform layer was separated and used as the lipid fraction. In a short time (~15 min) tracer experiments, cell collection and washing were omitted and lipids were extracted directly by adding 7.5 ml chloroform–methanol–water (1:2:8, by vol.) to 2 ml cell suspension. After adding 2 ml each of chloroform and water and shaking, the chloroform phase was used. The lipid fractions were evaporated, dissolved in chloroform and submitted to thin-layer chromatography with a solvent system of petroleum ether–ethyl ether–acetic acid.
(80:20:1) to separate lipid classes. For the determination of wax esters an aliquot of the chloroform solution was subjected to gas liquid chromatography (Yanaco, model GCG-550F) equipped with a hydrogen detector, on a glass column (100 x 0.3 cm) packed with 5% Thermon-100 at 230°C. Myristyl myristate or myristyl palmitate, prepared as in [8], was used as an internal standard.

The residue of the lipid extraction was dried and stirred in 5 ml 0.45 N perchloric acid. After centrifugation at 10000 x g for 10 min, the perchloric acid-soluble fraction was neutralized with potassium carbonate and the precipitate removed by centrifugation at 4°C. The supernatant was applied onto columns of Dowex 50 and 1 to separate amino acids, organic acids and sugars [9]. The perchloric acid-insoluble fraction was heated with 2 ml 1% SDS at 100°C for 15 min; the soluble and insoluble fractions were proteins and paramylon, respectively. Paramylon was solubilized with 1 N sodium hydroxide and determined by the phenol-sulfuric acid method [10]. Protein was determined as in [11]. CO₂ was trapped with 1 N potassium hydroxide.

For the determination of ATP, 0.1 ml 70% perchloric acid was added to the incubated cell suspension (2 ml) and cooled to 4°C. Cells were homogenized by sonication (10 kHz, 1 min) and the homogenate neutralized with potassium carbonate. After removing precipitate by centrifugation the supernatant was used for the ATP determination as in [12].

Radioactivity was measured with a liquid scintillation photometer (Aloka, model LSC-602).

3. RESULTS AND DISCUSSION

3.1. Formation of wax esters in anaerobiosis

The content of wax esters in E. gracilis (strain SM-ZK) grown to the stationary phase with shaking was 1 μg/10⁶ cells and, as shown in fig. 1, transferring the cells to anaerobiosis caused a prompt synthesis of wax esters with a concomitant decrease of the paramylon content. The synthesis of 39 μg/10⁶ cells of wax esters and loss of 105 μg/10⁶ cells of paramylon after 24 h of anaerobic incubation in the buffer correspond to 1.287 and 1.296 pmol/10⁶ cells, respectively, of acetyl-CoA, indicating that all carbons in the wax esters are supplied from paramylon. When the cells were brought back to aerobic conditions by bubbling air, the reverse changes took place; wax esters were decomposed rapidly while paramylon synthesized.

Fig. 2 shows distribution of radioactivity among CO₂, wax esters and polar lipids when aerobically grown Euglena, containing labelled paramylon (see section 2), was placed in anaerobiosis. Along with the prompt fall of radioactivity in paramylon, the activity in wax esters and CO₂ was increased, and the increased radioactivity in these fractions in sum was equivalent to more than 90% of the decreased radioactivity in paramylon; the ratio of radio
activity in wax esters and CO₂ was 2:1 at any time of incubation. The radioactivity in polar lipids and in other cell components was not changed by the transfer; no leak of radioactivity was detected into medium both from aerobic and anaerobic cells. The results suggest that CO₂ evolves by the action of pyruvate dehydrogenase in the course of glucose degradation and that acetyl-CoA simultaneously formed is utilized in the wax ester synthesis.

Aerobic Euglena cells were placed in anaerobiosis for different times, and then incubated anaerobically with [1-¹⁴C]acetate for 15 min. The activity of the cells to synthesize wax esters, as assayed by the incorporation of radioactivity into wax esters, increased rapidly with the adaptation period reaching a maximum after 15 min adaptation to anaerobiosis; the maximum activity was ~40-times higher than the activity in the aerobic cells. The addition of 2.5 µg cycloheximide/ml, the concentration effective to inhibit synthesis of some enzymes (unpublished), influenced neither the wax ester synthesis nor the paramylon degradation, indicating that the activation of wax ester synthesis does not involve an enzyme induction.

While the wax esters in aerobic cells are composed of saturated esters mainly with carbon number 28 and 30 and ~30% of unsaturated esters, those in anaerobic cells contain mainly saturated C28 ester with considerable amounts of saturated C26 and C27 esters but none of unsaturated ones.

3.2. Anaerobic ATP generation

Fig. 3 shows that placing aerobically grown Euglena cells in anaerobiosis causes a prompt fall in the cellular ATP level, but after 2 min, when the synthesis of wax esters becomes evident, the ATP level starts to increase gradually to regain the original level after 13 min, and this level is maintained during the anaerobiosis, showing that the synthesis of wax esters is coupled with the generation of ATP. Sodium arsenite and sodium fluoride, inhibitors of glycolysis, inhibited the ATP generation and wax ester synthesis in anaerobiosis.

The results heretofore obtained demonstrate that the phenomenon is a fermentation with wax esters as the end product, and is to be called the wax ester fermentation. ATP should be generated by the substrate-level phosphorylations in glycolysis. Thus, E. gracilis lives on respiration in the presence of sufficient amount of O₂ and on fermentation in anoxia by using paramylon as the reserve substance.

The fermentation with wax esters as the end product is unique in that they are with relatively high M₉-values and quite insoluble in water. They are sometimes called crystalloids [13], present as a suspending solid in cytoplasm, and apparently do not affect other metabolisms. Products of ordinary fermentations, lower alcohols and organic acids, are certainly water-soluble and may be harmful when accumulated in the cell so that they must be deposited with the loss of carbon. In Euglena the fermentation product is retained in the cell with as little a loss of carbon as possible, and when aerobic conditions are restored the wax esters are rapidly converted back to paramylon, and more efficient respiration takes place. The phenomenon resembles, in a sense, the lactate formation in anaerobic muscle and gluconeogenesis from lactate in aerobic liver in animals, but in unicellular Euglena the interconversion between glucan and wax esters takes place in the same cells.

3.3. Dependency of the energy metabolism on the O₂ concentration

An oxygen pump was employed to obtain N₂-O₂ mixtures with desired O₂ concentrations, and cells were adapted to different O₂ concentrations by bubbling cell suspension with the mixed
gases with different O\(_2\) concentrations for 15 min. The adapted cells were incubated with [2-\(^{14}\)C]pyruvate in potassium phosphate buffer for 15 min, and then the radioactivity in wax esters formed by fermentation and CO\(_2\) evolved by respiration was determined. The results are given in fig. 4. Along with the fall of O\(_2\) concentration, the radioactivity in wax esters was promptly increased at 10\(^{-5}\)-10\(^{-7}\) M while the radioactivity in CO\(_2\) promptly decreased at these concentrations. At <10\(^{-7}\) M O\(_2\) the incorporation of radioactivity into CO\(_2\) was negligible, and that into wax esters was constant. These data demonstrate that transition between respiration and fermentation in \textit{E. gracilis} occurs at 10\(^{-5}\)-10\(^{-7}\) M O\(_2\). The ATP level in \textit{Euglena} adapted to O\(_2\) at 10\(^{-3}\)-10\(^{-15}\) M, was quite identical.

In the degradation of paramylon in \textit{E. gracilis}, both \(\beta\)-1,3-glucanases and \(\beta\)-1,3-glucan phosphorylases are active [14], and depending on the extent of participation of these enzymes, net 2–3 molecules of ATP and 4 molecules of NADH are produced/molecule of glucose by glycolysis and the action of pyruvate dehydrogenase from paramylon. Acetyl-CoA produced from pyruvate is utilized for the synthesis of fatty acids and then wax esters. If the ordinary pathway of fatty acid synthesis involving malonyl-CoA intermediate is responsible for the wax ester synthesis in anaerobic \textit{Euglena} cells, the acetyl-CoA formed by pyruvate dehydrogenase located in mitochondria [15] must be translocated to cytoplasm, since bleached \textit{E. gracilis} contains the malonyl-CoA-involving system of fatty acid synthesis in cytoplasm [16–19] and microsomes [20,21]. In the course of translocation of acetyl-CoA and synthesis of malonyl-CoA, ATP is consumed, and therefore no net synthesis of ATP will ensue in the wax ester fermentation in \textit{Euglena}. It is probable that a novel system of fatty acid synthesis with little or no loss of ATP is operative in \textit{Euglena}, and this will be the subject of our forthcoming papers.

**REFERENCES**