NADPH-to-NADH conversion by mitochondrial transhydrogenase is indispensable for sustaining anaerobic metabolism in *Euglena gracilis*

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Most eukaryotes obtain energy by fermentation in anaerobic conditions due to decreased oxygen supply to the mitochondrial respiratory chain. Fermentation enables the production of two ATPs and two NADHs per glucose molecule by substrate-level phosphorylation in glycolysis. The NADH produced in glycolysis is regenerated to NAD$^+$ by the production of ethanol, acetate and lactate in the cytoplasm and is used in glycolysis again. On the other hand, some eukaryotes adapted to anaerobic environments, such as parasites, acquire energy through anaerobic respiration using molecules other than oxygen, e.g., fumarate, as the terminal electron acceptor in the mitochondrial respiratory chain [1,2].

In *Euglena gracilis*, a protist with chloroplasts, wax ester fermentation is primarily responsible for ATP synthesis under anaerobic conditions [3]. During the wax ester fermentation, paramylon, a storage polysaccharide (∂-1,3-glucan), is degraded, accumulating wax esters composed of medium- and long-chain saturated fatty acids and fatty alcohols (the end products) in the cell. In this process, acetyl-CoA is formed from pyruvate by the action of pyruvate:NADP$^+$ oxidoreductase in the mitochondria [4,5], where pyruvate is likely...

**Abbreviations**

MDH, malate dehydrogenase; ME, malic enzyme; NNT, nicotinamide nucleotide transhydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PNO, pyruvate: NADP$^+$ oxidoreductase.

*Euglena gracilis* produces ATP in the anaerobic mitochondria with concomitant wax ester formation, and NADH is essential for ATP formation and fatty acid synthesis in the mitochondria. This study demonstrated that mitochondrial cofactor conversion by nicotinamide nucleotide transhydrogenase (NNT), converting NADPH/NAD$^+$ to NADP$^+$/NADH, is indispensable for sustaining anaerobic metabolism. Silencing of NNT genes significantly decreased wax ester production and cellular viability during anaerobiosis but had no such marked effects under aerobic conditions. An analogous phenotype was observed in the silencing of the gene encoding a mitochondrial NADP$^+$-dependent malic enzyme. These results suggest that the reducing equivalents produced in glycolysis are shuttled to the mitochondria as malate, where cytosolic NAD$^+$ regeneration is coupled with mitochondrial NADPH generation.

**Keywords:** anaerobic respiration; *Euglena gracilis*; malic enzyme; transhydrogenase; wax ester fermentation
supplied from the glycolysis of paramylon-derived glucose in the cytosol. Then, fatty acids (acyl-CoA) are synthesized from acetyl-CoA by reversing β-oxidation in the mitochondria [6]. Half of these synthesized fatty acids are reduced to fatty alcohols, and are esterified with another half to form wax esters in microsomes [7,8]. Notably, in mitochondrial fatty acid synthesis, trans-2-enoyl-CoA acts as the terminal electron acceptor of the mitochondrial respiratory chain and is reduced to form acyl-CoA by the reverse reaction of acyl-CoA dehydrogenase (with FAD as a cofactor) using the electrons derived from mitochondrial respiratory chain complex I, which is coupled with anaerobic ATP synthesis [6].

In wax ester fermentation, therefore, the supply of NADH is of critical importance – NADHs are not only donating reducing power to complex I to drive the anaerobic respiratory chain but also reducing 3-ketoacyl-CoA by the reverse reaction of 3-hydroxacyl-CoA dehydrogenase in fatty acid synthesis. Notably, NADH is required to synthesize fatty acids in the mitochondria, in contrast to cytosolic fatty acid synthesis that uses NADPH. Nonetheless, only NADPH, not NADH, is generated in the anaerobic E. gracilis mitochondria, because the oxidative decarboxylation of pyruvate is catalysed by pyruvate: NADP+ oxidoreductase instead of common pyruvate dehydrogenase multienzyme complex (NAD+-dependent) [5]. Thus, we aimed to clarify how NADH was supplied in the anaerobic mitochondria.

In this study, we demonstrated that the nicotinamide nucleotide transhydrogenase (NNT) located in the mitochondrial inner membrane, presumably catalysing hydride transfer from NADPH to NAD+ and accompanying the proton translocation from the matrix to the intermembrane space [9], is indispensable for E. gracilis to synthesize wax esters to survive under anaerobic conditions. In addition, we observed that the mitochondrial NADP+-dependent malic enzyme (ME) played a pivotal role in importing glycolysis-derived reducing equivalents from the cytosol to the mitochondria, regenerating NAD+ in the cytosol to supplying additional NADPH in the mitochondria.

Materials and methods

Organism and culture

E. gracilis SM-ZK, a non-photosynthetic mutant strain [10], was cultured in Koren–Hutner medium with glucose as the major carbon source [11]. An aerobic culture was prepared by incubating the cells with reciprocal 120 r.p.m. shaking at 27 °C for 4 days to reach the late logarithmic growth phase. An anaerobic culture was prepared according to Nakazawa et al. (2018) [6] with slight modifications. In summary, a 3-mL aliquot of anaerobic-cultured cells was prepared in a 5-mL plastic tube and bubbled with argon gas for 1 min before the tube was tightly capped. Then, the cells were placed on a gyratory shaker at 8 r.p.m. at 27 °C for 48 h. Cell number and viability were determined according to Nakazawa et al. (2015) [12].

Chemical

UK-5099 (Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in DMSO and prepared as a 1000× solution (100 mM).

RNA interference

RNA interference experiments were conducted according to Nakazawa et al. (2015) [12]. A template DNA containing the sequence encoding T7 promoter at both ends was amplified from the E. gracilis cDNA preparation by PCR using specific primers (Table 1). The sequences of the mRNA encoding target genes were obtained using a TBLASTN search against an E. gracilis RNA-seq database (DRA accession number: SRP060591) [13]. The genes encoding the human mitochondrial NAD(P) transhydrogenase (UniProt accession ID Q13423) [14] and human mitochondrial NADP+-dependent ME (UniProt accession ID Q16798) [15] were used as the query sequences in the database search. Thirty micrograms of dsRNA was used in each experiment. The negative control experiments were conducted without the dsRNA.

Silencing of each target gene was verified using semi-quantitative reverse transcription (RT)-PCR according to Nakazawa et al. (2015) [12] using specific primers (Table 1). In addition, the expression of the genes was normalized to the level of the E. gracilis alpha-tubulin cDNA. Each 25-μL GoTaq Green Master Mix (Promega, Madison, WI, USA) PCR reaction mixture contained 0.4 μM of each primer and 0.5 μL of template cDNA. PCR amplification was carried out with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min kb⁻¹. Lastly, EgNNT1 was amplified for 27 cycles, EgME1 for 23 cycles and the alpha-tubulin gene for 19 cycles.

Measurement of transhydrogenase activity

Transhydrogenase activity was measured according to Phelps and Hatefi (1981) [16] with slight modifications. First, the cells were collected; suspended in 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂ and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated with a handy sonicator (UR-21P, Tomy Seiko, Japan) on ice for 15 s twice. Next, the
Table 1. Oligonucleotide primers used in this study. The T7 promoter sequence is underlined. Semi-qRT-PCR, semiquantitative RT-PCR; RNAi, RNA interference.

| Primer name          | Target cDNA  | Experiment     | Oligonucleotide (5’ to 3’)           |
|----------------------|--------------|----------------|--------------------------------------|
| EgNNT1-1121Fw        | EgNNT1       | Semi-qRT-PCR   | TGGACTGTGCCTGATGAGT                 |
| EgNNT1-1500Rv        | EgNNT1       | Semi-qRT-PCR   | GGAAGACTGAGTGCCGCTGCA               |
| EgME1-826Fw          | EgME1        | Semi-qRT-PCR   | CTGAGCTGCTGAAAAATCG                 |
| EgME1-1188Rv         | EgME1        | Semi-qRT-PCR   | CAGCTGAGCTGATCCAAACCAC             |
| EgAtub-853Fw         | alpha-tubulin| Normalizer     | AGAAGGCAGTACATGACAC                |
| EgAtub-1267Rv        | alpha-tubulin| Normalizer     | TCACCTCTCTGATACCCCTT                |
| T7-EgNNT1-99Fw       | EgNNT1       | RNAi           | CTAAATCGACTCTAGATTAGGGATTCAGTTCTCGTGGTCGCA |
| T7-EgNNT1-562Rv      | EgNNT1       | RNAi           | CTAAATCGACTCTATAGGGAGATTCTCTCTCTCGATTCAAGACAGCA |
| T7-EgME1-304w        | EgME1        | RNAi           | CTAAATCGACTCTATAGGGAGATTCTCTCGATTCAAGACAGCA |
| T7-EgME1-763Rv       | EgME1        | RNAi           | CTAAATCGACTCTATAGGGAGATTCTCTCGATTCAAGACAGCA |

crude extract containing mitochondrial membranes was obtained as the supernatant after centrifuging the cells at 500 × g for 1 min.

The NADPH→3-acetylpyridine adenine dinucleotide (AcPyAD) transhydrogenase activity was measured at 30 °C in a 245-μL reaction containing 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 0.5 mM NADPH, 1 mM AcPyAD and the crude extract. The reduction of AcPyAD was monitored for 5 min by measuring the absorbances at 375 and 425 nm using a Multiscan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reduction rate of AcPyAD was calculated using a value of 6.36 mm⁻¹ cm⁻¹ for the absorbance difference of AcPyADH and NADPH at the above wavelengths. One unit of transhydrogenase was defined as the amount of enzyme that catalysed the reduction of 1 nmol of AcPyAD per min.

Measurement of ME activity

NADP⁺-dependent malate decarboxylase activity of the ME was measured according to Karn and Hudock (1973) [17] with slight modifications. The cells were suspended in 30 mM potassium phosphate buffer (pH 7.5) and 1 mM PMSF, sonicated on ice for 15 s twice and centrifuged at 17,400 × g for 5 min. The supernatant was used as the crude cell extract. The reaction was conducted in a 1.5-mL mixture containing 6 mM potassium phosphate buffer (pH 7.5), 20 mM DL-malic acid, 10 mM MgCl₂, 0.5 mM NADP⁺ and the crude extract. The reduction of NADP⁺ was monitored for 5 min by measuring the absorbance at 340 nm using a U-2910 double-beam spectrophotometer (Hitachi High-Tech Science, Japan). The reduction rate of NADP⁺ was calculated using a value of 6.22 mm⁻¹ cm⁻¹. One unit of ME was defined as the amount of enzyme that catalysed the reduction of 1 nmol of NADP⁺ per min.

Analyses of wax esters

As described previously [12], lipids were extracted from E. gracilis cells and subjected to gas–liquid chromatography directly to determine the wax ester content.

Results

Effect of EgNNT1 silencing in the aerobic and anaerobic cultures

The TBLASTN search revealed two NNT orthologs in the E. gracilis RNA-seq database [13], and only one isozyme between them contained a putative mitochondrial targeting signal sequence at the N terminus (as predicted by a TargetP analysis [18]). Therefore, we designated this isozyme as a mitochondria-localized NNT candidate (EgNNT1; accession ID GDJR01019573.1). A TMHMM search [19] suggested that EgNNT1 contained at least 12 transmembrane domains. According to the localization of NNTs in other organisms [20], EgNNT1 was predicted to localize in the mitochondrial inner membrane.

Next, EgNNT1 was silenced by introducing double-stranded RNA. The degree of gene silencing was confirmed by semiquantitative RT-PCR (Fig. 1A) and by measuring EgNNT1 activity (Fig. 1B) in a 4-day aerobic culture. The activity of hydride transfer from NADPH to NADP⁺ in the crude extract was reduced by EgNNT1 silencing to less than 3% of the control. These results indicated that EgNNT1 was responsible for most of the NNT activity in E. gracilis. Despite the low NNT activity in the EgNNT1-silenced cells, aerobic growth was not affected by EgNNT1 silencing in the present culture conditions (Fig. 1C).

The effects of EgNNT1 silencing were more obvious under anaerobic than aerobic conditions. EgNNT1 silencing reduced the wax ester contents to ca. 28%
compared with the control cells after 48 h of anaerobic treatment (Fig. 1D). Anaerobic exposure also significantly decreased the viability of the EgNNT1-silenced cells, whereas no significant effect was observed in the control cells (Fig. 1E). These results suggest that EgNNT1 plays an essential role in the progression of wax ester synthesis and the survival of *E. gracilis* under anaerobic conditions.

**Effect of EgME1 silencing in the aerobic and anaerobic cultures**

Next, we focused on mitochondrial MEs, which enabled the cells to generate reducing equivalents in the mitochondria by the oxidative decarboxylation of malate presumably imported from the cytosol. We found a protein that exhibited significant homology to the NADP⁺-dependent ME with a mitochondrial targeting signal sequence (EgME1; accession ID GDJR01100105.1) according to the TBLASTN search and TargetP analysis.

*EgME1* was silenced by RNAi. The degree of gene silencing was confirmed by semiquantitative RT-PCR (Fig. 2A) and enzyme activity measurement (Fig. 2B). ME activity in the *EgME1*-silenced cells was decreased to below 10% compared with the control cells. These results suggested that EgME1 was responsible for most of the NADP⁺-dependent ME activity in *E. gracilis*. By contrast, aerobic growth was not affected by *EgME1* silencing (Fig. 2C).

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**Fig. 1. Effect of *EgNNT1* silencing on *Euglena gracilis* cells.** (A) Semiquantitative reverse transcription (RT)-PCR was performed to confirm *EgNNT1* silencing. In the control experiments, electroporation was conducted without dsRNA. The alpha-tubulin gene was used as the normalizer. Total RNA was prepared from the cells in a 4-day aerobic culture. (B) Determination of the hydride transfer activity from NADPH to NAD⁺ in the control (open bar) and *EgNNT1*-silenced cells (solid bar). Crude cell extracts were prepared from the cells in a 4-day aerobic culture. (C) The growth curves of the *EgNNT1*-silenced (closed circles with a solid line) and control (open circles with a dotted line) *E. gracilis* cells in aerobic culture. (D) Wax ester content after anaerobic exposure for 48 h. The control (open bar) and *EgNNT1*-silenced (solid bar) cells were aerobically cultured for 4 days and exposed to anaerobic conditions for 48 h. (E) Viability of the control (open bar) and *EgNNT1*-silenced (solid bar) *E. gracilis* cells before and after anaerobic exposure. Viability was determined by propidium iodide staining with fluorescent microscopy. *, a significant difference from the control cells (P < 0.05). The error bars indicate the standard deviation of the mean (n = 3).
The amount of wax ester in the cells was reduced to around 75% in the EgME1-silenced cells compared to the control cells after 48 h of anaerobic treatment (Fig. 2D). At the same time, the viability of the EgME1-silenced cells was greatly reduced compared to the control cells after 48 h of anaerobic exposure (Fig. 2E). These results suggest that EgME1 contributes significantly to anaerobic metabolism.

**Estimation of carbon transport pathways to the mitochondria using UK-5099, a pyruvate carrier inhibitor**

Then, we analysed how glycolysis-derived carbon was transported into the mitochondria under aerobic and anaerobic conditions by evaluating the physiological effects of UK-5099, an inhibitor of mitochondrial pyruvate carriers, on Euglena.

We examined the effect of UK-5099 on E. gracilis cells under aerobic conditions by adding UK-5099 at the beginning of culture and grew the E. gracilis cells aerobically in Koren–Hutner medium (containing glucose as the main carbon source) for 4 days. The growth of the E. gracilis cells was greatly reduced by adding UK-5099 (Fig. 3A), suggesting that pyruvate derived from glycolysis was transported into the mitochondria by a pyruvate carrier under aerobic conditions.

Next, we determined UK-5099’s effect on E. gracilis cells under anaerobic conditions. The amount of wax ester in the UK-5099-treated cells did not decrease compared with that in the control group after 48 h of anaerobic treatment (Fig. 3B). These results suggest
that the carbon produced by glycolysis is not transported to the mitochondria via a pyruvate carrier during anaerobiosis.

**Discussion**

In the anaerobic mitochondria of *E. gracilis*, ATP is provided by anaerobic respiration with fatty acid synthesis by  β-oxidation reversal. However, the mechanism of NADH supply in the mitochondria, necessary for both respiration and fatty acid production, remains unknown. By contrast, NADPH is produced in the mitochondria under anaerobic conditions by pyruvate: NADP⁺ oxidoreductase (PNO), which synthesizes acetyl-CoA to form the carbon chain of wax esters [5]. However, the reducing reaction consuming the NADPH during wax ester fermentation remains unknown. Therefore, we hypothesized that the conversion of NADPH to NADH in the mitochondria played an important role in sustaining anaerobic metabolism.

We focused on a transhydrogenase enzyme, *EgNNT1*, as an enzyme for NADPH-to-NADH conversion, and evaluated its role by silencing the gene encoding it. The silencing of *EgNNT1* substantially reduced wax ester synthesis and cell survival under anaerobic conditions (Fig. 1). NNTs are broadly conserved enzymes in prokaryotes and eukaryotes [20]. Furthermore, *EgNNT1* exhibited high homology to the proton-pumping NNTs of vertebrates localized in the inner mitochondrial membrane [21]. Therefore, in *E. gracilis*, it is supposed that protons are transported from the mitochondrial matrix to the intermembrane space with the conversion of NADPH to NADH by *EgNNT1*. Thus, *EgNNT1* should also contribute to ATP production coupled with anaerobic respiration.

Then, we focused on the mitochondrial NADP⁺-dependent ME, *EgME1*, because the coupling of mitochondrial NNT and ME has been reported in other organisms [22] and the ME reaction potentially supplies cytosol-derived reducing equivalents in the form of NADPH. Indeed, *EgME1* silencing resulted in *E. gracilis* cells with a similar phenotype to the *EgNNT1*-silencing cells (Fig. 2), indicating that *EgME1* contributes equally to the wax ester synthesis and cell survival of *E. gracilis* during anaerobiosis. Furthermore, as *EgME1* performs oxidative decarboxylation of malate to pyruvate, it supplies substrates, namely, malate, pyruvates, and NADPH, to PNO (pyruvate) and *EgNNT1* (NADPH), respectively. These data suggest that malate, instead of pyruvate, is transported from the mitochondria to the cytosol.
the cytosol to the mitochondria, as a result, reducing power generated in cytosolic glycolysis is imported to mitochondria under anaerobic conditions.

We, thus, investigated the effects of UK-5099, an inhibitor of mitochondrial pyruvate carriers, on pyruvate transport to the mitochondria in *E.gracilis* (Fig. 3). Contrary to the model of the conventional anaerobic metabolism in *Euglena*, the results showed that pyruvate carriers did not play any essential roles in supplying carbon to the wax ester synthesis system, whereas carbon was transported to mitochondria via the pyruvate carrier under aerobic conditions. Therefore, the findings support the idea that during anaerobic conditions, glycolysis-derived carbon is transported in as malate to shuttle cytosol-derived reducing equivalent into mitochondria. Together with a previous study which showed that *PNO*-silencing markedly reduced wax ester synthesis and survival during anaerobiosis [5], it is clear that NADPH supply and conversion to NADH in mitochondria are essential for both the synthesis of mitochondrial lipid production and energy acquisition by anaerobic respiration.

The wax esters produced by *E.gracilis* under anaerobic conditions contain odd-chain fatty acids and alcohols. Propionyl-CoA, a primer for odd-chain fatty acid synthesis, is produced in mitochondria from succinate via the methylmalonyl pathway [23]. In addition, when cultured anaerobically, succinate is secreted into the culture supernatant [24]. In anaerobic conditions, succinate is produced from malate via fumarate [23]. That is, malate is metabolized by at least two pathways in anaerobic *E.gracilis* mitochondria; one is conversion to acetyl-CoA by two oxidative decarboxylation reactions (catalysed by ME and PNO), another is conversion to succinate via fumarate reduction coupled with anaerobic respiration (mitochondrial complex II).

Malate dismutation (also known as the phosphoenolpyruvate carboxykinase (PEPCK)-succinate pathway) is an anaerobic mitochondrial metabolism in parasites. Malate dismutation consists of reactions that convert malate to acetate via pyruvate and succinate via fumarate [1,2,22,25–29]. It has been suggested that ME and NNT also function in parasitic systems. The anaerobic metabolism of *E.gracilis* appears to be a

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Fig. 4. A model of anaerobic metabolism in *E.gracilis* was inferred from this study and our previous study [6]. NADPH produced by PNO and ME1 under anaerobic conditions is converted to NADH by NNT1, and NADH is supplied (shown in dotted arrows) to mitochondrial complex I and fatty acid synthesis. One cycle of fatty acid β-oxidation oxidizes one molecule of NADH in the reverse reaction of 3-hydroxyacyl CoA dehydrogenase. PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; MDH, malate dehydrogenase; PNO, pyruvate:NADP⁺ oxidoreductase; RQ, rhodoquinone; ETF, electron transfer flavoprotein; ACD, acyl-CoA dehydrogenase.
variant of malate dismutation. PNO, which is absent in other parasites, allows \textit{E. gracilis} to obtain more NADPH and utilizes glucose-derived reducing equivalents more efficiently.

Figure 4 illustrates our new model of metabolism during anaerobiosis in \textit{E. gracilis}, inferred from previous studies \cite{6} and the results of the present study, where the contribution of PEPCK to wax ester synthesis \cite{30} and the presence of a cytosolic form of malate dehydrogenase (MDH) \cite{31} were considered. The anaerobic metabolic pathway via malate demonstrated in the present study agreed with the model pathway reported by Müller \textit{et al} \cite{1}. In anaerobic conditions, electrons generated in complex I are supplied to the wax ester synthesis system via rhodoquinone. As ubiquinone and rhodoquinone are present even in anaerobic \textit{E. gracilis}, it remains an open question how electron flow is regulated. It was suggested that a large part of paramylon-derived carbon is converted from phosphoenolpyruvate to malate via oxaloacetate and transported to the mitochondria. Existence of NNT enables both effective anaerobic energy acquisition and wax ester formation.

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Conflict of interest
The authors declare no conflict of interest.

Data accessibility
The data that support the findings of this study are available from the corresponding author [mami@biochem.osakafu-u.ac.jp] upon reasonable request.

Author contributions
All members conceived and designed the experiments and discussed the results for the completion of the manuscript. MN, MT, YM and RH performed the experiments and analysed the data. MN and HI wrote the manuscript.

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