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Physiological role of rhodoquinone in *Euglena gracilis* mitochondria

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Abstract

Rhodoquinone (RQ) participates in fumarate reduction under anaerobiosis in some bacteria and some primitive eukaryotes. *Euglena gracilis*, a facultative anaerobic protist, also possesses significant rhodoquinone-9 (RQ_9) content. Growth under low oxygen concentration induced a decrease in cytochromes and ubiquinone-9 (UQ_9) content, while RQ_9 and fumarate reductase (FR) activity increased. However, in cells cultured under aerobic conditions, a relatively high RQ_9 content was also attained together with significant FR activity. In addition, RQ_9 purified from *E. gracilis* mitochondria was able to trigger the activities of cytochrome bc_1 complex, bc_1-like alternative component and alternative oxidase, although with lower efficiency (higher K_m, lower V_m) than UQ_9. Moreover, purified *E. gracilis* mitochondrial NAD^+-independent D-lactate dehydrogenase (D-iLDH) showed preference for RQ_9 as electron acceptor, whereas L-iLDH and succinate dehydrogenase preferred UQ_9. These results indicated a physiological role for RQ_9 under aerobiosis and microaerophilia in *E. gracilis* mitochondria, in which RQ_9 mediates electron transfer between D-iLDH and other respiratory chain components, including FR.

Keywords: Microaerophilia; Rhodoquinone redox state; Fumarate reductase; Pyridine nucleotide-independent D-lactate dehydrogenase; Cytochrome bc_1 complex; Alternative oxidase

1. Introduction

Rhodoquinone (RQ; E°′ = −63 mV) is an essential component in fumarate reduction during anaerobic electron transfer in mitochondria of some primitive eukaryotic organisms such as *Ascaris suum* [1] and *Fasciola hepatica* [2] as well as in rhodobacteria [3]. Quinol fumarate reductases (QFR) in other types of bacteria use low potential menaquinone (E°′ = −74 mV) [4]. In *A. suum*, a correlation was observed between the RQ content and the rate of fumarate reduction in vivo. Mitochondria isolated from adult anaerobic *A. suum* diminish their cytochrome and ubiquinone (UQ) contents, whereas that of RQ substantially increases [1].

E. gracilis, a photosynthetic and facultative anaerobic protist, has a complex mitochondrial electron transfer chain constituted by typical respiratory enzymes (complexes I, II and IV) and a cytochrome bc_1 complex that is antimycin-sensitive but myxothiazol-resistant [5]. Alternative respiratory components are also present: NAD^+-independent L- and D-lactate dehydrogenases (L- and D-iLDH) [6], an alternative oxidase (AOX) [7], and bc_1-like activity (bc_1-*bypass*) sensitive to myxothiazol but antimycin-resistant [5]. Based on respiratory inhibition studies, the electron flux across the respiratory chain has been partially elucidated [5].

Rhodoquinone-9 (RQ_9) and ubiquinone-9 (UQ_9) are detected in *E. gracilis* [8]. Under aerobic growth conditions, RQ_9 is present in *E. gracilis* mitochondria with similar [7,8] or lower [9] concentration than UQ_9. On the other hand, under anoxic culture conditions the RQ_9 content increases whereas that of UQ_9 decreases in *E. gracilis* cells [9]. A significant concentration of RQ_9 is also found in mitochondria isolated from dark grown *E. gracilis*, which lacks chlorophyll and chloroplasts [7,8]. Moreover, an increase of both UQ_9 and RQ_9 is attained when cells are grown under cold or oxidative stress [7].

The last findings suggest that RQ in *E. gracilis* mitochondria might be engaged in other functions under aerobiosis, in addition to participating in fumarate reduction under anaerobiosis. Therefore, to elucidate the physiological relevance of RQ in *E. gracilis*, the effect of culturing under microaerophilic and aerobic conditions on the quinone pool and respiratory activities was analyzed.

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2. Materials and methods

2.1. Chemicals

Decylubiquinone (DBQ), ubiquinone-1 (UQ_1), ubiquinone-2 (UQ_2), ubiquinone-9 (UQ_9), DCPIP (2,6-dichlorophenol indophenol), antimycin, theonyltrifluoroacetone (TTFA), 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and methyl viologen were from Sigma Chem. Co. Rhodoquinone-9 (RQ_9) and rhodoquinone-10 (RQ_{10}) were purified from *Euglena gracilis* and *Rhodospirillum rubrum* as described below. Horse heart cytochrome *c*, n-propyl gallate (nPG) and myxothiazol were from ICN; stigmatellin was from Fluka, and isooctane from J. T. Baker.

2.2. Cell culture and preparation of mitochondria

Cells of *E. gracilis* Z were grown in the dark in the Hutner's acidic organotrophic medium with glutamate+malate as carbon source [10] at 25±1 °C under orbital shaking (125 rpm). Aerobic and microaerophilic cultures were inoculated with aerobic-cultured *E. gracilis* cells to a final concentration of 2×10^5 cells/ ml. The aerobic cultures contained 1 l of medium in 2-l Erlenmeyer flasks. Microaerophilic cultures were carried out culturing cells in 2 l Erlenmeyer flasks containing 2 l of medium previously bubbled with N_2 and then sealed and incubated without shaking.

Mitochondria were isolated as previously described [7] from cells reaching the stationary phase (96 h of culture). Mitochondrial protein was determined by the Biuret method as described elsewhere [11].

2.3. Cytochrome content

Reduced *minus* oxidized difference spectra of mitochondria was determined as previously described [7]. The content of cytochromes $a+a_3$, b and $c+c_1$ was estimated by using the extinction coefficients of $\varepsilon=16$ mM^{-1} cm^{-1} (609–630 nm) [12], $\varepsilon=25.6$ mM^{-1} cm^{-1} (563–578 nm) [13] and $\varepsilon=17.5$ mM^{-1} cm^{-1}(561–540 nm) [14], respectively.

2.4. Quinone analysis

Quinone-depleted mitochondria were obtained from lyophilized mitochondria that were incubated with isooctane for extraction of quinones according to the procedure previously described [7,15]. The quinones extracted from mitochondria were separated and identified by reverse-phase HPLC according to Wagner and Wagner [16]. Quinones were separated with a Waters C18 Spherisorb S5 ODS2 analytical column (4.6×250 mm, Waters PSS 831913) using nitrogen-purged ethanol/methanol (3:2 v/v) as the mobile phase. The flow rate was 1 ml/min. Detection of UQ and RQ was made at 275 and 283 nm, respectively. RQ_9 was purified from *E. gracilis* grown in aerobic conditions and RQ_{10} from *Rhodospirillum rubrum*. For RQ purification and identification, thin layer chromatography (TLC), UV-absorption spectrophotometry and infrared spectroscopy were used. To determine the redox state of the mitochondrial quinones under steady-state conditions, a rapid extraction with petroleum ether was used [16].

2.5. Enzyme activities

D-iLDH, L-iLDH and succinate dehydrogenase (SDH) activities in intact fresh mitochondria were determined as previously described [6] at room temperature in 1 ml of KME buffer (120 mM KCl, 20 mM MOPS (3-(N-morpholino)-propanesulfonic acid), 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), pH 7.3), 0.3 mM of DCPIP and 0.05–0.1 mg of mitochondrial protein. The reaction was started by adding L-lactate, D-lactate or succinate and the rate of DCPIP reduction was determined by measuring the absorbance change at 600 nm and using an extinction coefficient of $\varepsilon=21.3$ mM^{-1} cm^{-1} [17].

In contrast, activity of dehydrogenases in quinone-depleted mitochondria and that of purified mitochondrial D-iLDH were measured by following the change in absorbance of UQ or RQ at 275 and 283 nm, respectively. Extinction coefficients of $\varepsilon=12.5$ mM^{-1} cm^{-1} (from $E_{1\%1\ cm}=158$ at 283 nm) [18] for RQ and $\varepsilon=11$ mM^{-1} cm^{-1} (from $E_{1\%1\ cm}=141$ at 275 nm) [19] for UQ were used for calculations. The rate of quinone reduction was corrected for the remaining activity after adding 10 mM oxalate (L-iLDH, D-iLDH) or 10 mM malonate (SDH). These assays were carried out at 30 °C in 1 ml of KME buffer and with 0.015% triton X-100, when hydrophobic quinones were added; the reaction was started by adding the respective substrate. The purified D-iLDH enzyme solution contained 2.8% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 10 mM HEPES and 1 mM EGTA, pH 8 [20]. The amount of purified D-iLDH added in each assay was 50 μg protein (and 0.4% CHAPS final concentration). The purity of D-iLDH, determined by densitometric analysis of Coomassie blue stained SDS-PAGE [20], was approximately 60%. Incubation of enzyme with quinones for 2–10 min gave similar results; this indicated that the interaction of quinone-detergent micelles with the purified enzyme was relatively fast under the conditions assayed.

Fumarate reductase (FR) activity was measured at 30 °C using methyl viologen (MV) as electron donor ($\varepsilon=11.4$ mM^{-1} cm^{-1} at 600 nm) [21]. MV was reduced with dithionite until the absorbance reached a value of 0.8. The MV oxidation was monitored under a N_2 stream in the KME buffer using 0.3 mg protein; the reaction was started by adding 10 mM of freshly prepared fumarate. Non-enzymatic MV oxidation (<15%) was considered in the calculations.

Reduction of quinones with sodium borohydride and isolation of reduced quinones with cyclopentane was made as described elsewhere [22]. Reduced DBQ ($DBQH_2$), reduced UQ_9 (UQ_9H_2) and reduced RQ_9 (RQ_9H_2) (60 μM) were added to reconstitute electron transport, and oxygen uptake, in presence of triton X-100 (0.015%). A significant diminution of enzyme activities was obtained when sonication was used. Therefore quinones were incorporated into membranes by detergent solubilization.

Cytochrome *c* reductase activity was measured at 30 °C as previously reported [7] using quinone-depleted mitochondrial preparations. The reaction was started by adding 10 mM L-lactate or 60 μM $DBQH_2$, UQ_9H_2 or RQ_9H_2. Addition of 0.5 μM stigmatellin fully inhibited the reduction of cytochrome *c*. For the addition of hydrophobic quinones (UQ_9 and RQ_9), triton X-100 (0.015%) was also present. This detergent concentration yielded the highest activities; activity was negligible when reduced quinones were added in the absence of detergent. The cytochrome bc_1 complex and bc_1-*bypass* activities were estimated by adding 0.5 μM antimycin or 10 μM myxothiazol, respectively. Assays were also carried out with purified *E. gracilis* cytochrome bc_1 complex [23], using a protein concentration of 25–50 μg and incubated in 1 ml of KME buffer with 30 μM horse heart cytochrome *c* and 0.015% triton X-100, to allow solubilization of hydrophobic quinones. The activity of purified cytochrome bc_1 complex diminished (40%) by adding triton X-100, with no effect on K_m values for $DBQH_2$ and UQ_1H_2. The reaction was started by adding reduced quinone. The addition of 0.5 μM antimycin abolished the activity. An extinction coefficient for cytochrome *c* of $\varepsilon=19.1$ mM^{-1} cm^{-1} (550–540 nm) was used in the calculations [13].

The rate of respiration of fresh *E. gracilis* mitochondria was measured at 30 °C, with a Clark-type oxygen electrode, in an air-saturated KME buffer with 5 mM phosphate. Cyanide and nPG were added to evaluate the AOX capacity. TMPD oxidase activity was evaluated at 30 °C by determining the rate of O_2 uptake after adding 5 mM ascorbate plus 2.5 mM TMPD, which was fully inhibited by 10 mM azide or 1 mM cyanide. The oxygen solubility was determined to be 400 ng atoms/ml (200 μM) at 30 °C.

2.6. Determination of metabolites

L-lactate and D-lactate were determined by fluorometry at 30 °C according to standard methods [24]. Succinate was determined by measuring the amount of O_2 consumed by bovine heart or rat liver submitochondrial particles (2 mg protein) [25] at 25 °C in SHE medium (250 mM sucrose, 10 mM HEPES (4-(-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA, pH 7.3)+1 μM rotenone. This rate of O_2 uptake was fully inhibited by 5 mM malonate or 1 μM antimycin. The concentration of dissolved O_2 in the culture medium was estimated by using a Clark-type O_2 electrode.