THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 21, Issue of May 21, pp. 22422–22429, 2004
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Euglena gracilis Rhodoquinone:Ubiquinone Ratio and Mitochondrial Proteome Differ under Aerobic and Anaerobic Conditions*

Received for publication, January 28, 2004, and in revised form, March 5, 2004
Published, JBC Papers in Press, March 10, 2004, DOI 10.1074/jbc.M400913200

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***Euglena gracilis* cells grown under aerobic and anaerobic conditions were compared for their whole cell rhodoquinone and ubiquinone content and for major protein spots contained in isolated mitochondria as assayed by two-dimensional gel electrophoresis and mass spectrometry sequencing. Anaerobically grown cells had higher rhodoquinone levels than aerobically grown cells in agreement with earlier findings indicating the need for fumarate reductase activity in anaerobic wax ester fermentation in *Euglena*. Microsequencing revealed components of complex III and complex IV of the respiratory chain and the E1β subunit of pyruvate dehydrogenase to be present in mitochondria of aerobically grown cells but lacking in mitochondria from anaerobically grown cells. No proteins were identified as specific to mitochondria from anaerobically grown cells. cDNAs for the E1α, E2, and E3 subunits of mitochondrial pyruvate dehydrogenase were cloned and shown to be differentially expressed under aerobic and anaerobic conditions. Their expression patterns differed from that of mitochondrial pyruvate:NADP$^+$ oxidoreductase, the N-terminal domain of which is pyruvate:ferredoxin oxidoreductase, an enzyme otherwise typical of hydrogenosomes, hydrogen-producing forms of mitochondria found among anaerobic protists. The *Euglena* mitochondrion is thus a long sought intermediate that unites biochemical properties of aerobic and anaerobic mitochondria and hydrogenosomes because it contains both pyruvate:ferredoxin oxidoreductase and rhodoquinone typical of hydrogenosomes and anaerobic mitochondria as well as pyruvate dehydrogenase and ubiquinone typical of aerobic mitochondria. Our data show that under aerobic conditions *Euglena* mitochondria are prepared for anaerobic function and furthermore suggest that the ancestor of mitochondria was a facultative anaerobe, segments of whose physiology have been preserved in the *Euglena* lineage.**

* This work was supported by the Deutsche Forschungsgemeinschaft and BASF Plant Science (to W. M.) and by The Netherlands Organization for Scientific Research (A. G. M. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ620469, AJ620470, and AJ620471.

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Oxygen respiration, the most important process for ATP production in many eukaryotes, takes place in mitochondria. Its overall biochemistry, involving oxidative decarboxylation of pyruvate, citric acid cycle, electron transport chain, and oxidative phosphorylation, is conserved across many groups of fungi, higher plants, and animals (1), although the exceptions prove the rule: not all mitochondria require oxygen for ATP synthesis (2, 3). Numerous mitochondria synthesize ATP without the use of oxygen as terminal electron acceptor (2). Anaerobic mitochondria are found among unicellular eukaryotes (protists) (2, 4–6) and among various multicellular forms, such as parasitic worms (7–9), and marine animals like mussels (10). In some anaerobic mitochondria, ATP is synthesized via a proton-pumping electron transport chain, but different terminal acceptors, and alternative terminal oxidases accordingly, are used. Both external and endogenous terminal acceptors are used. Some fungi, for example *Fusarium oxysporum* and *Cylindrocarpon tonkinense*, can utilize nitrate or nitrite as terminal acceptor (5, 11). These mitochondria possess a nitrite reductase that reduces nitrite to nitrogen monoxide with electrons from the cytochrome *c* pool. An additional nitrate reductase can be used to reduce nitrate to nitrite, and the electrons for this reaction are derived from the ubiquinone pool (12). Mitochondria from parasitic worms such as *Fasciola hepatica* or *Ascaris suum* can use endogenously produced fumarate as their electron acceptor (2, 13). Reduction of fumarate by the action of fumarate reductase is directly coupled to electron transport and ATP synthesis but involves rhodoquinone instead of ubiquinone.

The single, reticulate mitochondrion of the flagellate *Euglena gracilis* is biochemically an intermediate between aerobic and anaerobic mitochondria. Several *Euglena* species adapt to a broad range of oxygen concentrations and can tolerate even very low concentrations of oxygen (14). *E. gracilis* can survive up to 6 months of oxygen deprivation in the dark with culturing on lactate (15). *Euglena* uses its mitochondrion for ATP synthesis in the presence and absence of oxygen (14, 16). Under aerobic conditions *Euglena* performs a more or less typical oxidative phosphorylation in association with a modified citric acid cycle and respiratory chain. Pyruvate from glycolysis enters the mitochondrion and is subjected to oxidative decarboxylation, but this is thought not to involve the typical mitochondrial pyruvate dehydrogenase (PDH)[1] complex but instead an

[1] The abbreviations used are: PDH, pyruvate dehydrogenase; UQ, ubiquinone; RQ, rhodoquinone; UQ_9, UQ-9; RQ_9, RQ-9; EST, expressed sequence tag; ESI-Q-TOF-MS/MS, electrospray ionization quadrupole time-of-flight tandem mass spectrometry; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; E1, pyruvate dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoyl dehydrogenase.

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unusual, oxygen-sensitive enzyme, pyruvate:NADP+ oxidoreductase (6, 17). The resulting acetyl-CoA enters a modified citric acid cycle, which entails a shunt via succinate semialdehyde as in the α-proteobacterium *Bradyrhizobium* (18), circumventing the step catalyzed by α-ketoglutarate dehydrogenase. Under anaerobic conditions, pyruvate:NADP+ oxidoreductase constitutes the key enzyme for a unique wax ester fermentation. Acetyl-CoA from decarboxylation of pyruvate serves in the absence of oxygen as the terminal acceptor of electrons from glucose oxidation whereby fatty acids are thought to be synthesized via an unusual reversal of β-oxidation that does not involve malonyl-CoA (19–21). A part of the fatty acids is reduced to alcohols, esterified with another fatty acid, and deposited in the cytosol as wax (hence wax ester fermentation). The stored waxes are degraded via aerobic dissimilation in the mitochondrion upon the return to aerobic conditions (19). Similar to the situation in anaerobic mitochondria of metazoa, wax ester fermentation in *Euglena* involves mitochondrial fumarate reduction and thus requires rhodoquinone (2), which was characterized from *Euglena* in early work (22).

To examine changes in mitochondrial biochemistry of *Euglena* during the shift from aerobic to anaerobic conditions, we investigated changes in ubiquinone and rhodoquinone content and isolated mitochondria from cells grown under both conditions to analyze their protein content via two-dimensional electrophoresis.

EXPERIMENTAL PROCEDURES

Medium and Culture Conditions—*E. gracilis* strain Z (SAG 1224–5/25 collection of algae Göttingen) for isolation of mitochondria and subsequent analysis by two-dimensional PAGE was cultured as described previously (6). *Euglena* cultures for determination of UQ_9 and RQ_9 were grown in a BIOSTAT B 10-liter fermenter (Braun Biotech) at a culturing volume of 7 liters with light continuously at 5000 lux, constant temperature at 28 °C, and stirring at 200 rpm. A defined medium as described by Ogbonna *et al.* (23) and Yamane *et al.* (24) was modified and used. One liter of medium contained 12 g of glucose, 0.8 g of KH_2PO_4, 1.5 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of $CaCO_3$, 0.0144 g of H_3BO_3, 2.5 mg of vitamin B_1, 20 μg of vitamin B_{12}, 1 ml of trace element solution, 1 ml of iron solution. The trace element solution contained 4.4 g of $ZnSO_4{\cdot}7H_2O$, 1.16 g of $MnSO_4{\cdot}H_2O$, 0.3 g of $Na_2MoO_4{\cdot}2H_2O$, 0.32 g of $CuSO_4{\cdot}5H_2O$, and 0.38 g of $CoSO_4{\cdot}5H_2O$/100 ml of distilled water. The iron solution contained 1.14 g of $(NH_4)_2SO_4Fe(SO_4)_2{\cdot}6H_2O$ and 1 g of EDTA/100 ml of distilled water. The pH of the medium was kept at 2.8 during the cultivation. Aerobic cultures were gassed with 2 liters/min air, and the anaerobic cultures were gassed with 2 liters/min nitrogen. Relative to O_2 levels in air-gassed uninoculated medium (set to 100%), anaerobic culture medium had 0% O_2 as determined with the BIOSTAT B electrode, although photosynthetic O_2 production was not specifically blocked by inhibitors in N_2-gassed, light-grown cultures. Cultures were harvested 5 days after inoculation with a starting density of 35,000 cells/ml. Cells were harvested by centrifugation and used directly (not frozen) for isolation of mitochondria or determination of UQ_9 and RQ_9 content.

Isolation of Mitochondria and Marker Enzymes—Spheroplast preparation by partial trypsin digestion of the pellicle and gentle mechanical disruption and fractionation by differential centrifugation was performed by the method of Chaudhary and Merrett (25). Mitochondria were purified on discontinuous Percoll gradients as described by Inui *et al.* (20). Marker enzyme activities of succinate-semialdehyde dehydrogenase (EC 1.2.1.16) for mitochondria (26), lactate dehydrogenase (EC 1.1.1.27) for the cytosol (27), and glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$) (EC 1.2.1.13) for chloroplasts (28) were determined using the assays described, respectively.

Two-dimensional Electrophoresis—Separation of mitochondrial proteins by two-dimensional PAGE was performed according to Görg *et al.* (29, 30). Isoelectric focusing was performed on a IPGphor isoelectric focusing system (Amersham Biosciences) according to the manufacturer's instructions. Up to 1 mg of mitochondrial protein was included in the rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer, bromphenol blue), and the IPG strip (18 cm, pH 3–10, Amersham Biosciences) was allowed to rehydrate at 20 °C for 12 h. Focusing was performed for 0.5 h at 100 V, 1 h at 500 V, 1 h at 1000 V, and 9 h at 8000 V. IPG strips were equilibrated for 30 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, bromphenol blue) with 1% (w/v) dithiothreitol and for 30 min in equilibration buffer with 3% (w/v) iodoacetamide followed by the transfer to the top of a 12% SDS-polyacrylamide gel and covering with agarose sealing solution (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 0.5% (w/v) agarose, bromphenol blue). SDS-PAGE was performed in a Hoefer SE 600 vertical electrophoresis unit (Amersham Biosciences) with a current of 40 mA/gel. Gels were stained with Coomassie by the method of Neuhoff *et al.* (31) or silver-stained as described by Blum *et al.* (32).

In-gel Digestion and ESI-Q-TOF-MS/MS Analysis—Protein spots of interest were cut from the gel, washed twice with 50% (v/v) acetonitrile, and incubated successively with 100% acetonitrile, 100 mM NH_4HCO_3, and 100 mM 1:1 NH_4HCO_3:acetonitrile. After vacuum drying, the gel pieces were reswollen with 10 ng μl^{-1} trypsin (Promega) and digested for 12 h at 37 °C. Peptides were extracted in 5% (v/v) formic acid using a sonication bath. Prior to mass spectrometry, samples were desalted using C18 ZipTips (Millipore). ESI-Q-TOF-MS/MS analysis of tryptic peptides was performed with a QSTAR XL mass spectrometer (Applied Biosystems).

Determination of UQ_9 and RQ_9 Content—Harvested *Euglena* cells were washed twice with phosphate-buffered saline, diluted to a final titer of 10^7/ml, and lyophilized. Lipids were extracted from these samples essentially according to Bligh and Dyer (33). After evaporation (nitrogen stream at 40 °C) of the organic phase, the lipid residue was dissolved in 15% (v/v) diethyl ether in hexane. Quinones were eluted from a silica column (LiChrolut Si 200) with 15% (v/v) diethyl ether in hexane, dried by a nitrogen stream, and dissolved in ethanol.

Quinones were separated on a reverse phase RP-18 column (LiChrospher, end-capped, 5 μm, 250 × 4.6 mm, Merck) using a linear gradient from 7 to 20% (v/v) diisopropyl ether in methanol with 0.1% (v/v) acetic acid in 24 min. Quinones were detected with a PE Sciex API 365 mass spectrometer equipped with an atmospheric pressure chemical ionization interface. Measurements were performed in the positive ionization mode. Quantification of eluted quinones was performed by selective reaction monitoring taking $M + H^+$ as a parent ion (795.6 for UQ_9 and 780.6 for RQ_9), and the specific product ion for UQ_9 and the specific product ion for RQ_9 were 197.1 and 182.1, respectively (Fig. 1). Calibration of the liquid chromatography mass spectrometry method was performed using UQ_9 standards (Sigma) and RQ_9 standards (isolated from *A. suum* according to Bligh and Dyer (33) and purified as described by Van Hellemond *et al.* (34)), which resulted in linear response curves between 0.1 and 100 pmol for RQ_9 and between 0.35 and 350 pmol for UQ_9. The concentrations of the UQ_9 and RQ_9 standards were spectrophotometrically determined using the following extinction coefficients: UQ_9, $E_{1\%\ 1\ cm} = 185$ at 275 nm (22); RQ_9, $E_{1\%\ 1\ cm} = 140$ at 283 nm (35).

Identification of PDH Subunits E1α, E2, and E3—Standard molecular methods, nucleic acid isolation, cDNA synthesis, and cloning in λ ZAPII were performed as described previously (36, 37). Hybridization probes for PDH subunits from *Euglena* were obtained by comparisons of in-house *Euglena* EST data with annotated sequences in the National Center for Biotechnology Information data base using BLAST. Oligonucleotides for screening a library constructed with mRNA from aerobically grown *Euglena* cells were designed as follows: PDH-E1α, 5′-AATCTCCTTGTCCACCTCCTTGTTAGTCTCCTTCTCGACTGCCTT-3′; PDH-E2, 5′-TTTCAAATCAGCATTGTAGACAACTGGGGTGATCAGTCCGTT-3′; PDH-E3, 5′-CCTTGGAAGGGGACAAGAAGGTGAAGGTCGTTGGAGAAACGA-3′.

Sequence Analyses—Data base searching, sequence handling, and alignment were performed with programs of the GCG package, version 10.3 (38). Reinspection of alignments and automatic exclusion of gaps were performed with programs clus2mol and rminsdel of the MOLPHY package, version 2.3 (39). Phylogenetic inference was performed using NeighborNet planar graphs (40) of protein LogDet distances (41); graphs were displayed with SplitsTree package, version 3.2 (42).

RESULTS

Isolation of Mitochondria—Purity of mitochondria isolated and purified from *Euglena* cells cultured aerobically or anaerobically was assayed by marker enzymes to exclude cross-contamination of the mitochondrial fraction with other cell compartments (Table I). Marker enzyme activities for chloroplasts were not detectable, and only very slight activities of cytosolic marker enzyme were measured (Table I). Succinate-semialdehyde dehydrogenase, the marker enzyme for *Euglena*

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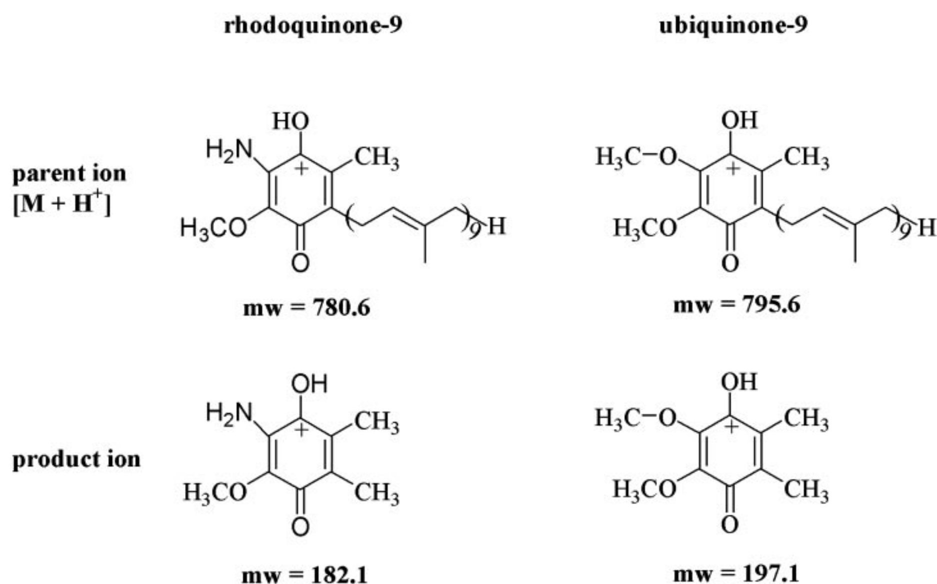

FIG. 1. **Mass spectrometry identification of UQ and RQ.** Parent ions [M + H^+] of ubiquinone-9 and rhodoquinone-9 and their specific product ions are shown. The liquid chromatography mass spectrometry selective reaction monitoring method was based on the appearance of these specific ions.

TABLE I
Marker enzyme activities

Activities are expressed in nmol·mg^{-1}·min^{-1} for crude extract and mitochondrial fraction of *E. gracilis* cells grown under aerobic and anaerobic conditions (±S.D.).

Enzyme	Crude extract, aerobic (n = 8)	Mitochondrial fraction	
		Aerobic (n = 8)	Anaerobic (n = 6)
Succinate-semialdehyde dehydrogenase	18.8 ± 5.8	173 ± 22.5	153 ± 13.2
Glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$)	431 ± 15.6	0	0
Lactate dehydrogenase	17,950 ± 1570	2 ± 0.8	6 ± 0.7

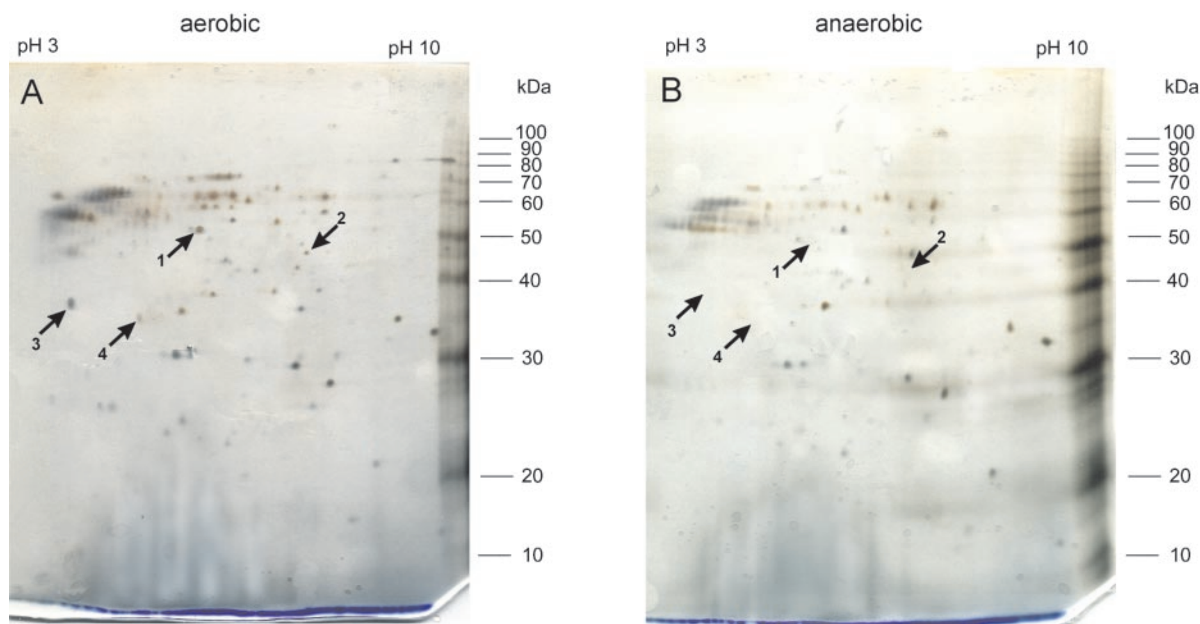

FIG. 2. **Mitochondrial proteome analysis.** Silver-stained two-dimensional polyacrylamide gel loaded with 800 μg of mitochondria isolated from aerobically (*A*) and anaerobically (*B*) grown *E. gracilis* cells. *Numbered spots* showed the same pattern of expression in three independent culture/mitochondria isolation experiments and correspond to proteins indicated in Table II. *Arrows* in *B* indicate the corresponding positions of *spots 1–4* labeled in *A*.

mitochondria, was strongly enriched in the mitochondrial fraction as compared with the crude extract (Table I).

Separation of Mitochondrial Proteins by Two-dimensional PAGE—Mitochondrial proteins from aerobic and anaerobic cells separated in the first dimension in pH 3–10 gradients and subsequent separation on SDS-PAGE were compared. The overall number of protein spots observed here for *Euglena* mitochondria is rather small in comparison with recent studies of mitochondrial proteomes in yeast or mouse (43, 44). However, our focus here is on major proteins involved in core energy metabolism whose levels change in response to the transition to anaerobiosis. No new protein spots appeared during the shift from aerobic to anaerobic culturing conditions; rather some spots present in aerobic mitochondria disappeared under an-

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TABLE II
Identification of protein spots

Spots were identified from two-dimensional PAGE of isolated mitochondria from aerobically grown *E. gracilis* cells by mass spectrometry sequencing of tryptic peptides.

Spot no.	Sequenced peptides	Protein function	Identification
1	VLEQLLGSSYS, FDGTTNLADDLGR, VPLASFFEQLDALSR, ADLWVGVT. . . .TLR, VQEQEDVEAR	Ubiquinol-cytochrome *c* reductase complex core protein I	GenBank™
2	SAIAFTVEGFR, DGLTSSEYITK, GSPLGHTSFVPAYNLGYIDSNK, SAALLTAYGNVESWR, AKEFDDQFTDVYSTYTAYAFK, ATQATLIDSFNTTGQPLSPLEIVSAIK	Ubiquinol-cytochrome *c* reductase complex core protein II	GenBank™
3	DLQQVAAFS, QTLTEYALLEGQNQLVQR, VNDFVDSNPVYL, TALACLNA. . . .NDVDALR	Cytochrome *c* oxidase subunit IV	EST data
4	QNEAAGLSA, SGGQLQ, SPWNAED	Pyruvate dehydrogenase E1 β-subunit	GenBank™

TABLE III
Ubiquinone and rhodoquinone content of Euglena cells

Quinones were extracted and determined as described under "Experimental Procedures." Means of three independent aerobic and anaerobic cultures are shown (±S.D.). The average protein content of the *Euglena* cells was 0.34 ± 0.06 ng/cell.

Growth conditions	RQ_9 content	UQ_9 content	RQ:UQ ratio	RQ (percentage of total quinone content)
	nmol/mg protein	*nmol/mg protein*		*%*
Aerobic	0.12 ± 0.03	0.31 ± 0.05	0.38 ± 0.04	28 ± 2
Anaerobic	0.18 ± 0.026[a]	0.24 ± 0.02[a]	0.77 ± 0.15[b]	43 ± 5[b]

[a] $p < 0.2$.
[b] $p < 0.05$.

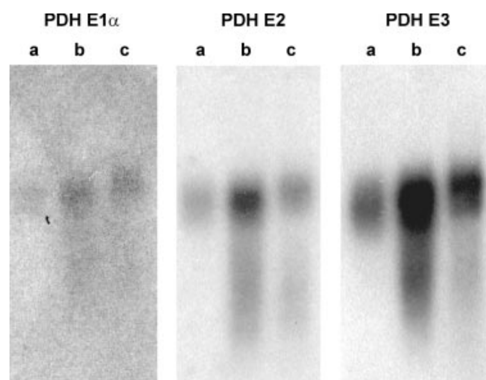

FIG. 3. **Gene expression under different conditions.** Shown are the Northern blots of *E. gracilis* poly(A)$^+$ mRNA (5 μg/lane) extracted from cells grown as follows: air with 2% CO_2 and light (*a*), N_2 with 2% CO_2 and light (*b*), N_2 with 2% CO_2 in the dark (*c*). The blots were probed with PDH E1α, PDH E2, and PDH E3 as indicated. The band of PDH E1α is 1.6 kb, and the bands of PDH E2 and PDH E3 are 1.8 kb; no additional bands were detected.

aerobic conditions (Fig. 2). Spots identified as aerobic specific spots in mitochondria from three independent cultures were excised from Coomassie-stained gels, digested with trypsin, and analyzed by ESI-Q-TOF-MS/MS. Sequenced peptides were compared with GenBank™ with options for short nearly exact matches and by searching *Euglena* EST data. Three sequenced spots that are missing in mitochondria from anaerobically grown cells belonged to components of electron transfer in the mitochondrial respiratory chain (Fig. 2, *spots 1*, *2*, and *3*). These three spots represented two components of mitochondrial complex III (ubiquinol-cytochrome *c* reductase complex core protein I and complex III core protein II) plus one component of complex IV (cytochrome *c* oxidase subunit IV). In addition, analysis of a fourth spot indicated that the E1β subunit of the PDH complex (Fig. 2, *spot 4*) was also not expressed under anaerobic conditions (Table II).

Identification and Cloning of PDH Subunits E1α, E2, and E3—Comparisons of our *Euglena* EST data with public data bases via BLAST revealed EST sequences with strong sequence similarity to the pyruvate dehydrogenase (E1α), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3) subunits of mitochondrial pyruvate dehydrogenase complex; cDNAs for these PDH subunits were isolated and found to encode proteins of 379, 434, and 474 amino acids, respectively. Northern hybridization revealed that all three PDH subunits are expressed under both aerobic and anaerobic conditions. Messenger RNA expression levels in anaerobically light-grown cells were about 2-fold higher than those in anaerobically dark-grown cells, whereas aerobically grown cells had reduced mRNA levels in comparison to anaerobically gassed cells grown in the light (Fig. 3).

Determination of Ubiquinone and Rhodoquinone Content—Rhodoquinone and ubiquinone content was determined in aerobically and anaerobically grown cells. RQ and UQ concentrations differed between aerobically and anaerobically grown *Euglena* cells (Table III). In anaerobically grown cells, a decrease in ubiquinone content was detected compared with aerobically grown cells, whereas these anaerobic cells showed an increase in rhodoquinone content. Consequently the RQ:UQ ratio was increased in anaerobically grown *Euglena*. In anaerobically grown *Euglena*, RQ was 43% of the total quinones (RQ + UQ) compared with 28% under aerobic conditions. The total amount of quinones did not differ between aerobically and anaerobically grown cells.

DISCUSSION

Proteomic Studies Reveal an Anaerobic Response in Euglena Mitochondria—In the mitochondrion of *E. gracilis* α-ketoglutarate is converted via α-ketoglutarate decarboxylase to succinate semialdehyde, which is further oxidized to succinate by succinate-semialdehyde dehydrogenase (14), the marker enzyme for *Euglena* mitochondria. By the criteria of marker enzymes, isolated *Euglena* mitochondria used for two-dimensional PAGE analysis were free from contaminating cell fractions (Table I).

In the comparison of mitochondrial proteins from aerobically and anaerobically grown cells, no major spots were detected that were anaerobic specific, but several proteins were detected that are present in aerobic cells yet missing under anaerobic conditions. That no proteins were observed to accumulate *de novo* in *Euglena* mitochondria upon the shift from aerobic to anaerobic conditions (Fig. 2) suggests that the enzymes necessary for anaerobic ATP synthesis are already present under aerobic conditions and that these enzymes become physiologi-

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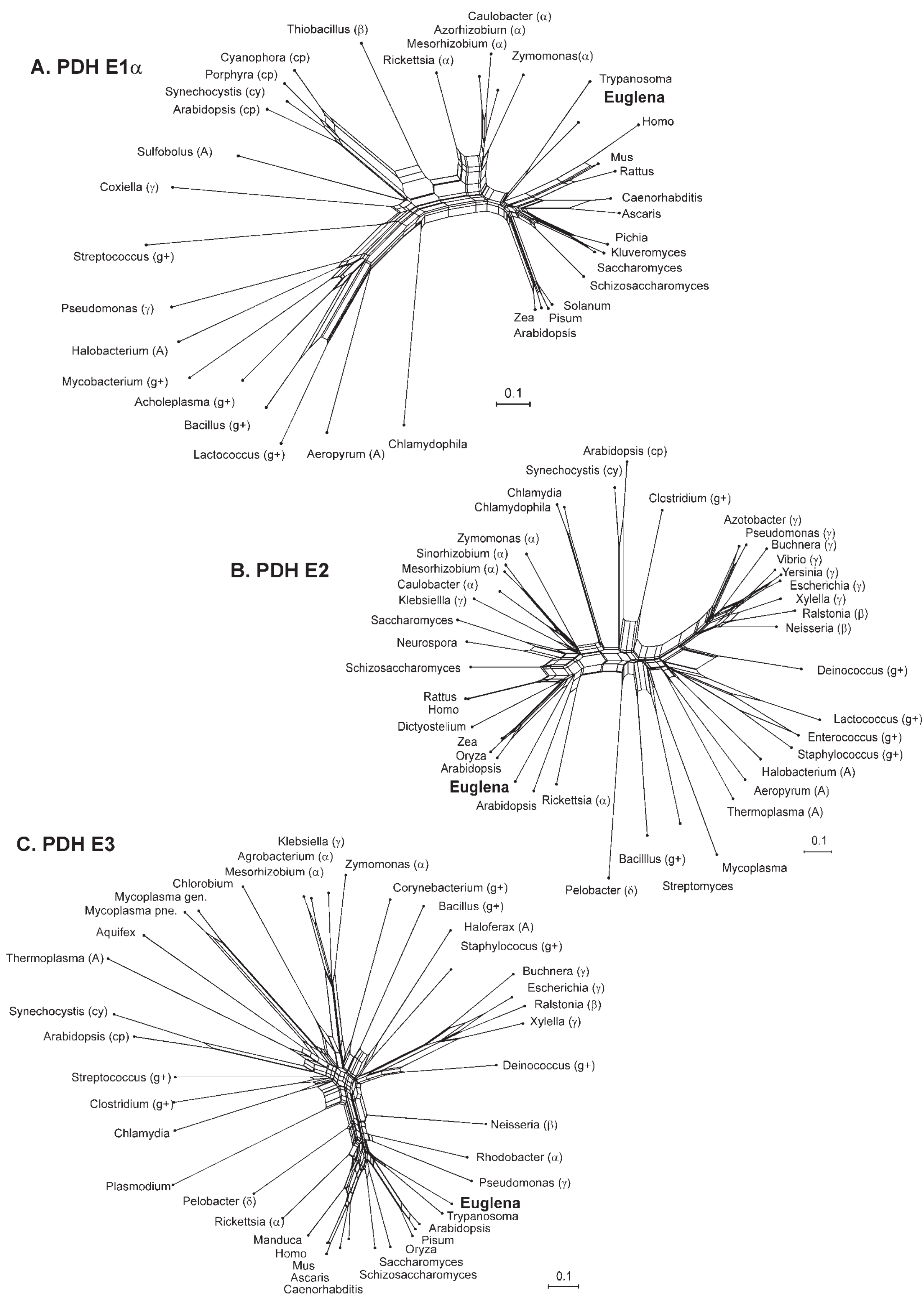

FIG. 4. **Patterns of PDH sequence similarity.** NeighborNet planar graphs of protein LogDet distances among PDH subunits are shown. Splits (bifurcations, branches) in the data are indicated as series of *parallel lines*. For example, the *Caenorhabditis* PDH E1α sequence shares strong similarity to the homologue from *Ascaris* but also shares a conflicting component of similarity with the homologues from *Homo*, *Mus*, and *Rattus*

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cally relevant at the moment when oxygen is no longer available as an electron acceptor. This kind of "metabolic readiness" in mitochondrial energy metabolism is known for some parasitic worms. For example, the liver fluke *F. hepatica* (45) uses fumarate as the terminal acceptor in the absence of oxygen. The fumarate reductase and rhodoquinone that are required anaerobically are also present in the aerobic stages of the life cycle of this parasite but are not used in the presence of oxygen (2, 13). *Fasciola* mitochondria are thus prepared for anaerobiosis before it is encountered (7, 45). The present findings that no mitochondrial proteins were observed to be anaerobic specific suggest that *Euglena* may follow a similar strategy: "be prepared for anaerobiosis."

We identified two components of mitochondrial respiratory chain complex III and one component of complex IV that are down-regulated under anaerobic conditions (Fig. 2 and Table II). These results are in agreement with earlier data from Carre *et al.* (15) who measured the disappearance of cytochrome oxidase and cytochrome c_{558} in *E. gracilis* under prolonged culture with anoxic conditions. Upon the return to aerobic conditions, *Euglena* first depends upon a cyanide-resistant electron pathway (15), which is in agreement with our results that components of complex III and complex IV are missing under anaerobic conditions. The cyanide-resistant, alternative respiratory pathway is known for higher plants, many algae, fungi, and certain protozoa. It branches from the standard mitochondrial electron transfer chain (cytochrome pathway) at the level of the quinone pool (46, 47). This alternative pathway includes the presence of an alternative oxidase that is distinguished from cytochrome *c* oxidase by its insensitivity to cyanide, azide, and carbon monoxide (48). A partial cDNA encoding a homologue of eukaryotic mitochondrial alternative oxidase has been identified among our *Euglena* ESTs (data not shown). We did not observe any components of complex I or complex II to be down-regulated under anaerobic conditions. Thus, in light of previous findings, our present results suggest that in aerobic conditions *Euglena* mitochondria possess the necessary enzymes and cofactors (rhodoquinone) required for anaerobic redox balance and ATP synthesis and that under anaerobic conditions aerobic specific components are no longer synthesized whereby upon return to aerobic conditions the alternative, cyanide-insensitive oxidase, using oxygen as terminal electron acceptor, may maintain redox balance in the transition stage.

Euglena Mitochondria: Pyruvate Dehydrogenase and Pyruvate:NADP$^+$ Oxidoreductase—In addition to components of complex III and IV, we found the E1β subunit of pyruvate dehydrogenase to be down-regulated under anaerobic conditions at the protein level (Fig. 2 and Table I). The other three subunits of PDH were identified in our EST data base, and full-length clones were obtained by screening a cDNA library. Evidence for an expressed PDH protein in *Euglena* mitochondria was somewhat surprising because this canonical mitochondrial activity is usually reported as non-existing in *Euglena* with the oxygen-sensitive enzyme pyvuvate:NADP$^+$ oxidoreductase substituting for it instead (17, 49, 50). However, in one early and previously isolated study, PDH activity in *Euglena* mitochondria was measured (51). In support of that finding, we have found the mRNAs for three PDH subunits and have found the fourth (E1β) through protein sequencing in isolated mitochondria.

Pyruvate dehydrogenases are generally organized as large multienzyme complexes that include multiple copies of three different enzymes. The E1 component contains pyruvate dehydrogenase (EC 1.2.4.1) activity, E2 is a dihydrolipoyl transacetylase (dihydrolipoyllysine-residue acetyltransferase, EC 2.3.1.12), and E3 is a dihydrolipoyl dehydrogenase (EC 1.8.1.4) (52). In eukaryotes, regulatory components such as PDH kinase, phospho-PDH phosphatase, and E3-binding protein are also commonly associated with the mitochondrial PDH complex (53). These accessory proteins are apparently lacking in bacterial PDH where regulation occurs through allosteric mechanisms and product inhibition (54). The E1 protein of PDH from mitochondria and from Gram-positive bacteria is composed of two different subunits (E1α and E1β), which form an $\alpha_2\beta_2$ heterotetramer (52, 55). In contrast, the E1 protein in many Gram-negative bacteria is organized as a homodimer of translationally fused α and β subunits ($(\alpha\beta)_2$) (54, 55). Our present data from *Euglena* indicate that its mitochondrial PDH has a typical E1α, E1β, E2, E3 subunit organization as in other eukaryotes. Additionally we found *Euglena* ESTs with 42% amino acid identity over 104 residues (*E* value, $<10^{-16}$) to mitochondrial PDH kinase from *Schizosaccharomyces pombe* and other eukaryotes (data not shown), suggesting the existence of typical eukaryotic regulatory components for *Euglena* PDH as well.

Although mitochondrial pyruvate:NADP+ oxidoreductase from *E. gracilis* is expressed under aerobic and anaerobic conditions (6), the present findings reveal that it coexists with a classical PDH in the organelle. Northern hybridization (Fig. 3) shows that E1α, E2, and E3 PDH subunits show expression levels that are converse to that of pyruvate:NADP+ oxidoreductase under the aerobic and anaerobic conditions tested because pyruvate:NADP+ oxidoreductase showed weakest expression under N_2 in the light, stronger expression under air in the light, and highest expression under N_2 in the dark (6), whereas PDH E1α, E2, and E3 mRNA levels are highest under N_2 in the light, lower under N_2 in the dark, and lowest under air in the light (Fig. 3). Sequence comparisons of *Euglena* PDH subunits to their homologues from eukaryotes and prokaryotes reveal that they cluster with mitochondrial and α-proteobacterial homologues (Fig. 4), indicating a common inheritance from the mitochondrial symbiont. A distinct advantage of the NeighborNet (40) planar graph representation of the sequence similarities over a bifurcating tree is that conflicting data and poorly resolved relationships are directly depicted, providing a more conservative interpretation of sequence similarities and clusters. In other words, rather than showing all of the conflicting bifurcating trees that would be compatible with the data, the graphs show the conflicting signals contained within the data in a single diagram.

Rhodoquinone Levels in Euglena Change with Oxygen Availability—In wax ester fermentation in *Euglena* mitochondria, the synthesis of even-numbered fatty acids starts from acetyl-CoA, whereas odd-numbered fatty acid synthesis starts from propionyl-CoA, which is generated via succinate, propionate, and methylmalonyl-CoA (21, 56, 57). The formation of propionyl-CoA involves fumarate reductase, which catalyzes the reverse reaction of that in succinate dehydrogenase (complex II) but requires the lower midpoint potential of rhodoquinone *versus* ubiquinone to function efficiently in the succinate-synthesizing direction (2, 13, 58, 59). Rhodoquinone and ubiquinone

to the exclusion of the *Ascaris* homologue (a conflicting phylogenetic signal). The *scale bar* at the lower right side indicates estimated substitutions per site. Sequences were retrieved from GenBank™ and from finished and unfinished genome projects through The Institute for Genomic Research and the National Center for Biotechnology Information. *A*, PDH E1α sequences. *B*, PDH E2 sequences. *C*, PDH E3 sequences. Abbreviations indicated in parentheses are as follows: α, β, γ, and δ, proteobacteria; *g+*, Gram positives; *cy*, cyanobacteria; *cp*, plastid-specific isoform; *A*, archaebacteria.

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content in aerobic and anaerobic *Euglena* cultures revealed significant differences. In anaerobically grown *Euglena* cells, rhodoquinone comprised 43% of total quinones as compared with 28% in aerobically grown cells (Table III). The necessity of rhodoquinone for an electron transport chain involving fumarate reduction has been shown for eukaryotes in general (34). The increase of rhodoquinone content in anaerobically grown *Euglena* cells is consistent with the increased flux through fumarate reductase in the synthesis of odd-numbered fatty acids in wax ester fermentation, and the presence of rhodoquinone aerobically is consistent with the view that aerobically grown *Euglena* is prepared for an instantaneous switch to anaerobic conditions.

Anaerobic and Aerobic Biochemistry in One Organelle—From an evolutionary standpoint, the *Euglena* mitochondrion has interesting biochemical features. It contains proteins usually specific to hydrogenosomes (the pyruvate:ferredoxin oxidoreductase domain of pyruvate:NADP+ oxidoreductase (6)) and typical of mitochondria (PDH). This can be taken as further evidence to indicate that mitochondria and hydrogenosomes are simply different specializations of one and the same ancestral organelle (60). Indeed the family of mitochondrial organelles encompasses many specialized members, including forms that do not make ATP for the cell at all but make iron-sulfur clusters instead (61). An intriguing aspect of the *Euglena* mitochondrion is that it is not fully specialized to either aerobic or anaerobic environments but is able to function under both conditions just like many contemporary members of the α-proteobacteria can (62). Furthermore it contains ubiquinone and rhodoquinone, which are specific to aerobic and anaerobic mitochondrial functions, respectively (2), whereby both quinone types are also both found among contemporary α-proteobacteria (63). Although the rhodoquinone-utilizing fumarate reductases of anaerobic mitochondria and α-proteobacteria arose independently (2, 64), it is not yet clear whether the ability to synthesize rhodoquinone is a direct inheritance from the ancestor of mitochondria or whether it evolved independently (2) because the biochemistry of rhodoquinone synthesis is not yet known.

Newer geochemical evidence indicates that the oceans of the earth were anoxic and furthermore had high concentrations of sulfide up until about 1–0.6 billion years ago (65, 66). Since the early diversification of eukaryotic lineages occurred prior to 1 billion years ago (67), early eukaryotes must have been able to survive recurrent anoxic conditions. Biochemical relics of that anaerobic past are preserved in various eukaryotic lineages today (2, 60) and are often (but not always) associated with mitochondrial function and/or localized in mitochondria, for example iron-only hydrogenase (60), sulfide:quinone oxidoreductase (68), or the alcohol dehydrogenase E that is otherwise typical of amitochondriate protists (69, 70). Anaerobic mitochondria and hydrogenosomes are unexplained in traditional formulations of the endosymbiont hypothesis, which posit a strictly aerobic lifestyle for the ancestor of mitochondria and convolute suggestions for the origins of hydrogenosomes (71, 72). An alternative model suggests the origin of both aerobic and anaerobic biochemical pathways in mitochondria and hydrogenosomes to be an inheritance from a facultatively anaerobic eubacterial common ancestor of both organelles (73). Comparative studies of eukaryotes and α-proteobacteria that can survive in anaerobic environments should provide further insights about the nature of the creature that became the mitochondrion.

Acknowledgment—We gratefully acknowledge the help of Dr. Sabine Metzger at the Biologisch-Medizinisches Forschungszentrum of the University of Düsseldorf in protein sequencing.

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